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(54) Title: DIRECT ADMINISTRATION OF GENE DELIVERY VEHICLES AT MULTIPLE SITES

(57) Abstract

Methods of stimulating an immune response, either humoral or cell-mediated, in a warm-blooded animal through the administration at multiple sites of one or more gene delivery vehicles is provided. Each of the gene delivery vehicles directs the expression of at least one substance in host cells modified with the vehicle, such that an immune response is generated. Within preferred embodiments, the expressed substance elicits a cell-mediated immune response, preferably an HLA Class I-restricted immune response.

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Direct administration of gene delivery vehicles at multiple sites

Technical Field

The present invention relates generally to the administration of gene delivery vehicles to warm-blooded animals at multiple sites, in order to stimulate an immune response in the animal.

Background of the Invention

Although bacterial diseases are, in general, easily treatable with antibiotics, very few effective treatments or prophylactic measures exist for many viral, cancerous and other nonbacterial diseases. Traditional attempts to treat these diseases have employed the use of chemical drugs. In general, these drugs have lacked specificity and have exhibited high overall toxicity.

Another classic technique for treating a number of nonbacterial diseases involves the elicitation of an immune response to a pathogenic agent, such as a virus, through the administration of a nonpathogenic form of the agent, such as a killed virus, thereby providing antigens from the pathogenic agent which would confer specific immunity.

A more recent approach for treating viral diseases, such as acquired immunodeficiency syndrome (AIDS) and related disorders, involves blocking receptors on cells susceptible to infection by HIV from receiving or forming a complex with viral envelope proteins. For example, Lifson et al. (*Science 232*:1123-1127, 1986) demonstrated that antibodies to CD4 (T4) receptors inhibited cell fusion (syncytia) between infected and noninfected CD4 presenting cells *in vitro*. A similar CD4 blocking effect using monoclonal antibodies has been suggested by McDougal et al. (*Science 231*:382-385, 1986). Alternatively, Pert et al. (*Proc. Natl. Acad. Sci. USA 83*:9254-9258, 1986) have reported the use of synthetic peptides to bind T4 receptors and block HIV infection of human T-cells, while Lifson et al. (*J. Exp. Med. 164*:2101, 1986) have reported blocking both syncytia and virus/T4 cell fusion by using a lectin which interacts with a viral envelope glycoprotein, thereby blocking it from being received by CD4 receptors.

Another recently suggested technique for inhibiting a pathogenic agent, such as a virus, which transcribes RNA is to provide antisense RNA, which is complementary to and binds at least a portion of the transcribed RNA, so as to inhibit translation (To et al., *Mol. Cell. Biol. 6*:758, 1986).

However, a major shortcoming of the techniques described above is that they do not readily lend themselves to control as to the time, location or extent to which the drug, antigen, blocking agent or antisense RNA are utilized. In particular, since the above techniques require exogenous application of the treatment agent (*i.e.*, exogenous to the sample in an *in vitro* situation), they are not directly responsive to the presence of the pathogenic agent. For example, it may be desirable to have an immunostimulant expressed in increased amounts immediately following infection by the pathogenic agent. In addition, in the case of antisense RNA, large amounts would be required for useful therapy in an animal, which, given current techniques, is extremely difficult to administer to the location at which it is actually needed, that is, at the cells infected by the pathogenic agent.

As an alternative to exogenous application, techniques have been suggested for producing treatment agents endogenously. More specifically, proteins have been expressed from viral vectors based on DNA viruses, such as adenovirus, simian virus 40, bovine papilloma virus, and vaccinia virus. By way of example, Panicali et al. (*Proc. Natl. Acad. Sci. USA 80*:5364, 1983) introduced influenza virus hemagglutinin and hepatitis B surface antigens into the vaccinia genome and infected animals with the virus particles produced from these recombinants. Following infection, the animals acquired immunity to both the vaccinia virus and the hepatitis B antigen.

However, a number of difficulties have been experienced to date with vectors based on DNA viruses. These difficulties include (a) the production of viral proteins which may lead to pathogenesis or the suppression of the desired protein; (b) the capacity of the vector to uncontrollably replicate in the host, and the pathogenic effect of such uncontrolled replication; (c) the presence of wild-type virus which may lead to viremia or rescue of the recombinant virus; and (d) the transitory nature of expression in these systems.

Each of the commonly used vector systems based on DNA viruses have also presented particular problems, which limit their usefulness as gene delivery vehicles. For example, because of the ubiquitous nature of adenoviruses, rescue of a wild-type adenovirus by recombination is a possibility. As well, prevalent preexisting immunity potentially renders an individual refractory to infection or may lead to a seriously damaging immune response. In this regard, a recent cystic fibrosis patient receiving therapy using an adenovirus vector presented with a damaging inflammatory response. Likewise, vaccinia virus is a highly immunogenic virus, making repeated treatments with a vaccinia virus vector difficult. Moreover, the demonstrated toxicity of proteins produced by vaccinia virus in immunocompromised individuals limits the usefulness of these viral vectors (Redfield et al., *New England Journal of Medicine 316*:673, 1987). Two other DNA viruses, herpes simplex virus (HSV) and adeno-associated virus (AAV) have more recently

been exploited for vector development. However, each of these two viruses has had limited success as a vector system due in part to the toxicity of residual viral proteins produced by these vectors, and like vaccinia virus and adenovirus, preexisting host immunity to these viruses further limits their applicability.

In an attempt to circumvent the difficulties associated with DNA virus vectors, physical gene transfer vectors have been proposed. These vectors include naked DNA, liposome-assisted gene transfer, and polycation-assisted gene transfer. Although obviating some of the problems outlined above associated with DNA virus vectors, these physical gene transfer vectors suffer from their own set of difficulties. Limitations affecting their usefulness include transient expression of introduced genes, a low efficiency of transfection or the inability to transfect a large number of cells, manufacturing complexity making large-scale production problematic, and the elicitation of an immune response to various components of the complexes.

Due to the nontransitory nature of their expression in infected target cells, retroviruses have been suggested as a useful vehicle for the treatment of genetic diseases (for example, *see* F. Ledley, *The Journal of Pediatrics 110*:1, 1987). In this regard, long-term expression from retroviral vectors, which is necessary for the treatment of genetic diseases, has been demonstrated for factor IX (Dai et al., *Proc. Natl. Acad. Sci. USA 89*:10892, 1992) adenosine deaminase (Van Beusechem et al., *Proc. Natl. Acad. Sci. USA 89*:7640, 1992; Blaese, *Pediatr. Res. 33* (supplement):549, 1993), and β-globin (Plavec, *Blood 81*:1384, 1993). In addition to genetic diseases, other researchers have contemplated using retroviral vectors to treat nongenetic diseases (*see, for example,* EP 243,204 - Cetus Corporation; Sanford, *J. Theor. Biol. 130*:469, 1988; Tellier et al., *Nature 318*:414, 1985; and Bolognesi et al., *Cancer Res. 45*:4700, 1985). Expression of genes designed to elicit an immune response, such as the HIV *env* gene in treatment of AIDS (Jolly et al., *J. Cell Biochem. 18A* (Suppl.):220, 1994), thymidine kinase expression followed by gancyclovir treatment to irradicate brain tumors (Culver et al., *Science 256*:1550, 1992), and γ-IFN expression to treat virus infections (Howard et al., *Proc. Natl. Acad. Sci.*, 716:167-187, 1994) have been proposed and are being tested.

The present invention provides an improvement over the previously suggested use of such gene delivery vehicles for the stimulation of an immune response, and further provides other related advantages.

Summary of the Invention

Briefly stated, the present invention is directed toward methods of stimulating an immune response, either humoral or cell-mediated, through the

administration to a warm-blooded animal at multiple sites of one or more gene delivery vehicles, each gene delivery vehicle directing the expression of at least one substance in host cells modified (*i.e.*, transduced, transfected or transformed) with the vehicle, such that an immune response is generated. In one aspect, the present invention provides gene delivery vehicles which direct the expression of at least one antigen or modified form thereof in host cells infected or transfected with the gene delivery vehicle, the antigen or modified form thereof being capable of stimulating an immune response within an animal. Within preferred embodiments, the expressed substance elicits a cell-mediated immune response, preferably an HLA class I-restricted immune response.

Within the context of HIV infection, the gene delivery vehicle preferably directs the expression of a substance which will elicit both an HLA class I- and class II-restricted immune response. In the case of HIV envelope antigen, for example, the antigen is preferably selected from gp 160, gp 120 and gp 41, which have been modified to reduce their pathogenicity. In this regard, the antigen may be modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, and to remove immunodominant, but strain-specific epitopes, and yet allow an immune response capable of eliminating cells infected with most or all strains of HIV (*see* WO 91/02805).

Similarly, in the context of FIV infection, the gene delivery vehicle preferably directs the expression of at least one immunogenic portion of a feline immunodeficiency virus antigen. Suitable antigens in this regard include p15gag, p24gag, p10gag, p13pol, p62pol, p15pol and p36pol. Within a particularly preferred embodiment, gene delivery vehicles are provided which direct the expression of gp68env, gp27env and rev. Within the context of the present invention, "rev" is understood to refer to the antigen corresponding to the rev open reading frame (*see* Phillips et al., First International Conference of Feline Immunodeficiency Virus Researchers, University of California, Davis, CA, September 4-7, 1991).

Further, in the context of feline leukemia virus (FeLV) infections, the gene delivery vehicle preferably directs the expression of at least one immunogenic portion of an FeLV antigen selected from p15gag, p12gag, p27gag, p10gag, p14pol, p80pol, p46pol, gp70env and p15env. Within a particularly preferred embodiment, gene delivery vehicles are provided which direct the expression of gp85env.

Within the context of hepatitis infection, the gene delivery vehicle preferably directs the expression of HBeAg, HBcAg, HBsAgs, ORF 5, ORF 6, the HBV pol antigen, or any combination of these antigens (*e.g.*, HBeAg and HBcAg). Within one embodiment, the HBsAgs are selected from the group consisting of S, pre-S1, and pre-S2.

Within yet another embodiment of the invention, gene delivery vehicles are provided which direct the expression of an immunogenic portion of antigen X, or co-express this antigen with an immunomodulatory factor. Within related embodiments, the gene delivery vehicle directs the expression of the hepatitis C core antigen, antigen E1, antigen E2/NS1, antigen NS2, antigen NS3, antigen NS4, antigen NS5, or combinations thereof. Within still another embodiment, gene delivery vehicles are provided which direct the co-expression of at least one immunogenic portion of a hepatitis B antigen and at least one immunogenic portion of a hepatitis C antigen. Within another related aspect of the invention, gene delivery vehicles are provided which direct the expression of an immunogenic portion of a polyprotein antigen, or co-express this antigen with an immunomodulatory factor.

Within another aspect of the present invention, gene delivery vehicles are provided which direct the expression of at least one immunogenic, non-tumorigenic form of an altered cellular component. Within the context of the subject invention, "altered cellular component" refers to proteins and other cellular constituents which are either associated with rendering a cell tumorigenic, or are associated with tumorigenic cells in general, but are not required or essential for rendering the cell tumorigenic. Before alteration, the cellular components may be essential to normal cell growth and regulation, and include for example, proteins which regulate intracellular protein degradation, transcriptional regulation, cell-cycle control, and cell-cell interaction. After alteration, the cellular components no longer perform their regulatory functions, and hence the cell may experience uncontrolled growth. Within various embodiments, the cellular component may be altered by a point mutation, by a deletion, or by a chromosomal translocation. Within other embodiments, the altered cellular components include, ras*, p53*, Rb*, altered protein encoded by the Wilms' tumor gene, ubiquitin*, mucin*, DCC, APC, MCC, neu, an altered receptor, or polypeptides resulting from chromosomal translocations such as bcr/abl. Within another embodiment, non-tumorigenic altered cellular components are provided, including for example, Δ ras*12, Δ ras*13, and Δ ras*61. Also provided are gene delivery vehicles which direct the expression of several altered cellular components, including, for example, a gene delivery vehicle which directs the expression of both ras* and p53*, or a gene delivery vehicle which directs the expression or ras*, mucin*, and DCC.

Within another aspect, gene delivery vehicles are provided which direct the expression of at least one immunogenic non-tumorigenic form of a normal cell component associated with the tumorigenic state (*i.e.*, a tumor associated antigen or TAA). Suitable components in this regard include antigens such as MART 1, MAGE 1 and 3, and tyrosine hydroxylase.

In addition to directing the expression of substances that stimulate an immune response, the gene delivery vehicles can be used to specifically immunosuppress certain responses by the immune system. T cell activation requires the interaction of the antigen specific T-cell receptor with antigen presented on the surface of antigen presenting cells (APC) and secondary signals delivered by the interaction of costimulatory molecules on the surface of T-cells (CD4, CD8, LFA-1, CD2, CD3, CD28, CTLA4, etc.) and APC (MHC II, MHC I, ICAM-1, ICAM-2, LFA-3, B7.1, B7.2, B7.3, etc.). By blocking the secondary interaction of CD28 or CTLA4 with B7.1, B7.2, or B7.3, the particular T cell is rendered non-responsive, even to future encounters with antigen. Within this context, a gene delivery vehicle encoding an allergen such as casein, glutin, bee venom or albumin, an autoantigen such as myelin basic protein or pancreatic β cells or a foreign antigen present on donor graft tissue such as MHC encoded alloantigens and antisense and/or ribozyme sequences for down-regulation of B7.1, B7.2, and B7.3 is administered at multiple sites to induce a specific immunosuppression to a particular antigen. In addition, gene delivery vehicles encoding an allergen, an autoantigen or a foreign antigen present on donor graft tissue may be co-injected with a second gene delivery vehicle encoding soluble CTLA4, CD28, or B7.1-.3 in order to induce specific immunosuppression to a particular antigen.

Within the context of the present invention, a wide variety of gene delivery vehicles may be utilized, including for example viral vectors, nucleic acid vectors with or without transfection enhancers such as local anesthetics, liposomes or polycation condensing agents, and bacteria. Suitable viral vectors include recombinant retroviruses or a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus (*e.g.*, the canary pox virus or the vaccinia virus), influenza virus, adenovirus, adeno-associated virus (*e.g.*, the adeno-associated virus, B19 or MVN), herpes virus, SV40, HIV, measles, alpha viruses such as Sindbis virus or corona virus.

The gene delivery vehicle may be administered at multiple sites to a patient directly, for example by direct injection, or alternatively, through the use of target cells transduced *ex vivo*. The present invention also provides pharmaceutical compositions (including, for example, various adjuvants) suitable for administering the gene delivery vehicles.

These and other aspects of the present invention will become evident upon reference to the following detailed description and attached drawings.

Brief Description of the Drawings

Figure 1A is a graph depicting the results of a CTL assay performed on animals injected with a representative gene delivery vehicle at multiple sites.

Figure 1B is a graph depicting the results of a CTL assay comparable to Figure 1A performed on animals injected with the same representative gene delivery vehicle at a single site.

Figure 2A is a graph depicting the results of a CTL assay performed on animals injected with a representative gene delivery vehicle at multiple sites.

Figure 2B is a graph depicting the results of a CTL assay comparable to Figure 2A performed on animals injected with the same representative gene delivery vehicle at a single site.

Detailed Description of the Invention

Prior to setting forth the invention, it may be helpful to an understanding thereof to first define certain terms that will be used hereinafter. All references which have been cited herein are hereby incorporated by reference in their entirety.

"Immunogenic portion" as utilized within the present invention refers to a portion of the respective antigen which is capable, under the appropriate conditions, of causing an immune response (*i.e.*, cell-mediated or humoral). "Portions" may be of variable size, but are preferably at least 8 amino acids long, and may include the entire protein molecule. Representative assays which may be utilized to determine immunogenicity (*e.g.*, cell-mediated immune response), are described in more detail below. Cell mediated immune responses may be mediated through Major Histocompatability Complex ("MHC") class I presentation, MHC class II presentation, or both.

"Gene delivery vehicle" refers to an assembly which is capable of directing the expression of the sequence(s) or gene(s) of interest. The gene delivery vehicle will generally include promoter elements and may include a signal that directs polyadenylation. In addition, the gene delivery vehicle includes a sequence which, when transcribed, is operably linked to the sequence(s) or gene(s) of interest and acts as a translation initiation sequence. The gene delivery vehicle may also include a selectable marker such as Neo, SV_2 Neo, TK, hygromycin, phleomycin, histidinol, or DHFR, as well as one or more restriction sites and a translation termination sequence. In addition, if the gene delivery vehicle is a retrovirus, a packaging signal and long terminal repeats (LTRs) appropriate to the retrovirus used will be included(if these are not already present). Further, if the gene delivery vehicle is a Sindbis virus, the vehicle RNA will include a 5' sequence which is capable of initiating transcription, as well as sequences which, when expressed, code for biologically active Sindbis non-structural proteins (*i.e.*, NS1, NS2, NS3, and NS4). In addition, the Sindbis gene delivery vehicle should include a Sindbis RNA polymerase recognition sequence, and a viral junction region, which may, in certain embodiments, be

modified in order to either prevent or inhibit viral transcription of the subgenomic fragment. The gene delivery vehicle may also include nucleic acid molecule(s) which are of a size sufficient to allow production of viable virus, as well as one or more restriction sites. When the Sindbis vector genome is transfected as a DNA molecule to give a eucaryotic layered vector system (ELVS), it should additionally include a 5' RNA polymerase II promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination and splice recognition.

"Immunomodulatory factor" refers to factors which, when manufactured by one or more of the cells involved in an immune response, or, which when added exogenously to the cells, causes the immune response to be different in quality or potency from that which would have occurred in the absence of the factor. The quality or potency of a response may be measured by a variety of assays known to one of skill in the art including, for example, *in vitro* assays which measure cellular proliferation (*e.g.*, ^{3}H thymidine uptake), and *in vitro* cytotoxic assays (*e.g.*, which measure ^{51}Cr release) (*see*, Warner et al., *AIDS Res. and Human Retroviruses 7*:645-655, 1991). Immunomodulatory factors may be active both *in vivo* and *ex vivo*. Representative examples of such factors include cytokines, such as interleukins 2, 4, 6, 12 and 15 (among others), alpha interferons, beta interferons, gamma interferons, GM-CSF, G-CSF, and tumor necrosis factors (TNFs). Other immunomodulatory factors include, for example, CD3, ICAM-1, ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules, B7.1-.3, b_2-microglobulin, chaperones, or analogs thereof.

Briefly, immunomodulatory factors can be produced in a number of known ways (Ellis and Gerety, *J. Med. Virol. 31*:54-58, 1990), including chemical synthesis (Bergot et al., *Applied Biosystems Peptide Synthesizer User Bulletin No. 16, 1986*, Applied Biosystems, Foster City, California) and DNA expression in recombinant systems, such as the insect-derived baculovirus system (Doerfler, *Current Topics in Immunology 131*:51-68, 1986), mammalian-derived systems (such as CHO cells) (Berman et al., *J. Virol. 63*:3489-3498, 1989), yeast-derived systems (McAleer et al., *Nature 307*:178-180), and prokaryotic systems (Burrel et al., *Nature 279*:43-47, 1979).

As noted above, the present invention is directed toward the administration of gene delivery vehicles at multiple sites in order to stimulate an immune response, preferably a cell-mediated immune response. Briefly, the ability to recognize and defend against foreign pathogens is central to the function of the immune system. This system, through immune recognition, is capable of distinguishing "self" from "non-self" (foreign) and attacking non-self, which is essential to ensure that defensive mechanisms are directed towards invading entities rather than against host tissues. This specificity is provided by clonotypic T cell receptors (TCR) present on each mature T cell. The TCR recognizes

specific peptide fragments of antigens displayed on a self major histocompatibility complex (MHC) encoded class I or class II molecule, the basis of MHC class I and II restriction. With some exceptions, each T cell has specificity for one peptide antigen displayed on one, self MHC molecule. To adequately protect the host animal from an almost infinite number of potential foreign antigens, the immune system relies on a large repertoire of antigen specificities that, when antigen is encountered, can expand and mobilize to mount an effective immune response. A second protective mechanism is that T and B cells of the immune system demonstrate long-term memory after encountering antigen, which is the principle of vaccination. These memory lymphocytes respond more rapidly and effectively to a second antigen encounter than the primary exposure. The methods which are described in greater detail below provide an effective means of inducing potent class I and II restricted, protective, and therapeutic T and B cellular and humoral immune responses.

Within the context of the present invention, the phrase "multiple sites" is equivalent to two or more sites. In this regard, at least two of the sites are associated with or involve alternate lymphoid tissues (*e.g.*, different lymph nodes, spleen, tonsils, bone marrow, thymus, liver). Within the context of the present invention, it will be evident that the antigen expressed via the gene delivery vehicles generally traffics centrally to the nearest lymph node, following the pathway for lymphatic or blood circulation. For example, antigen injected intramuscular to the deltoid muscle (or any upper arm site) will normally traffic to the axillary lymph nodes, while injection to the thigh or buttocks traffics to the inguinal lymph nodes. Similarly, intraperitoneal injection should traffic to the mesenteric lymph nodes and the spleen. By comparison, intravenous injection of gene delivery vehicles should traffic primarily to the spleen, and next to several lymph nodes. Intradermal and subcutaneous injection proceeds similar to intramuscular injections, in that antigen travels centrally to the nearest lymph node. Administration orally or sublingually generally traffics to the mesenteric lymph nodes, while oral, nasal, rectal, and vaginal administration of antigen generally travels to the nearest central lymph node. Given the routing of antigen as described herein, one can readily predict which lymph nodes will be involved. In addition, the precise lymph node involved may be confirmed through a lymph node biopsy, preferably in association with a diagnostic method (*e.g.*, fluorescent beads, PCR). Within preferred embodiments of the present invention, the gene delivery vehicles are administered immediately following one another up to 12 hours apart, with an interval of less than 1 hour being particularly preferred. Where several different gene delivery vehicles and/or routes of administration are utilized, it will be evident that the timing between administrations need not be uniform, but rather may be staggered or even random within the overall time of administration.

Gene Delivery Vehicles

Within the present invention, a variety of gene delivery vehicles which direct the expression of one or more heterologous nucleotide sequences are provided. "Gene delivery vehicles" as used within the present invention refers to recombinant vehicles, such as viral vectors (Jolly, *Cancer Gen. Therapy 1*:51-64, 1994), nucleic acid vectors, naked DNA, cosmids, bacteria, and certain eukaryotic cells (including producer cells;), that are capable of eliciting an immune response within an animal. Representative examples of such gene delivery vehicles include poliovirus (Evans et al., *Nature 339*:385-388, 1989; and Sabin, *J. Biol. Standardization 1*:115-118, 1973); rhinovirus; pox viruses, such as canary pox virus or vaccinia virus (Fisher-Hoch et al., *PNAS 86*:317-321, 1989; Flexner et al., *Ann. N.Y. Acad. Sci. 569*:86-103, 1989; Flexner et al., *Vaccine 8*:17-21, 1990; U.S. Patent Nos. 4,603,112, 4,769,330, and 5,017,487; WO 89/01973); SV40 (Mulligan et al., *Nature 277*:108-114, 1979); retrovirus (U.S. Patent No. 4,777,127, GB 2,200,651, EP 0,345,242, and WO 91/02805); influenza virus (Luytjes et al., *Cell 59*:1107-1113, 1989; McMicheal et al., *N. Eng. J. Med. 309*:13-17, 1983; and Yap et al., *Nature 273*:238-239, 1978); adenovirus (Berkner, *Biotechniques 6*:616-627, 1988; Rosenfeld et al., *Science 252*:431-434, 1991; WO 93/9191; Kolls et al., *PNAS 91*:215-219, 1994; Kass-Eisler et al., *PNAS 90*:11498-11502, 1993; Guzman et al., *Circulation 88*:2838-2848, 1993; Guzman et al., *Cir. Res. 73*:1202-1207, 1993; Zabner et al., *Cell 75*:207-216, 1993; Li et al., *Hum. Gene. Ther. 4*:403-409, 1993; Caillaud et al., *Eur. J. Neurosci. 5*:1287-1291, 1993; Vincent et al., *Nat. Genet. 5*:130-134, 1993; Jaffe et al., *Nat. Genet. 1*:372-378, 1992; and Levrero et al., *Gene 101*:195-202, 1991); parvovirus such as adeno-associated virus (Samulski et al., *J. Vir. 63*:3822-3828, 1989; Mendelson et al., *Virol. 166*:154-165, 1988; PA 7/222,684); herpes (Kit, *Adv. Exp. Med. Biol. 215*:219-236, 1989); SV40; HIV (Poznansky, *J. Virol. 65*:532-536, 1991); measles (EP 0 440,219); astrovirus (Munroe, S.S. et al., *J. Vir. 67*:3611-3614, 1993); Semliki Forest Virus, and coronavirus, as well as other viral systems (*e.g.*, EP 0,440,219; WO 92/06693; U.S. Patent No. 5,166,057). In addition, viral carriers may be homologous, non-pathogenic(defective), replication competent virus (*e.g.*, Overbaugh et al., *Science 239*:906-910,1988), and nevertheless induce cellular immune responses, including CTL.

In addition, an immune response (including CTL) may also be generated by administration of a bacterium which expresses the immunogenic portion(s) on its cell surface. Representative examples include BCG (Stover, *Nature 351*:456-458, 1991), salmonella (Newton et al., *Science 244*:70-72, 1989) and *E. coli*. (Cline et al., *Pharmac. Ther. 29*:69, 1985, and Friedmann et al., *Science 244*:1275, 1989).

Naked DNA or nucleic acid molecules are also suitable for use as gene delivery vehicles within the present invention (WO 90/11092). Such gene delivery vehicles

may be either DNA or RNA and, in certain embodiments, are linked to killed adenovirus (Curiel et al., *Hum. Gene. Ther. 3*:147-154, 1992). Other suitable vehicles include DNA-ligand (Wu et al., *J. Biol. Chem. 264*:16985-16987, 1989), lipid-DNA combinations (Felgner et al., *Proc. Natl. Acad. Sci. USA 84*:7413 7417, 1989), liposomes (Wang et al., *Proc. Natl. Acad. Sci. 84*:7851-7855, 1987) and microprojectiles (Williams et al., *Proc. Natl. Acad. Sci. 88*:2726-2730,1991).

In addition, one can increase the efficiency of naked DNA uptake into cells by coating biodegradable latex beads with naked DNA. This approach takes advantage of the fact that latex beads, when incubated with cells in culture, are efficiently transported and concentrated in the perinuclear region. The beads will then be transported into cells when injected into muscle. DNA coated latex beads will be efficiently transported into cells after endocytosis is initiated by the latex beads, and thus increase the gene transfer and expression efficiency of this method. This method may be improved further by treatment of the beads to increase their hydrophobicity and thereby facilitate the disruption of the endosome and release of the DNA into the cytoplasm.

In addition, *ex vivo* procedures may be used in which cells are removed from an animal, modified, and placed into the same or another animal. Cells that can be modified include, but are not limited to, fibroblasts, endothelial cells, hepatocytes, epithelial cells, lymphocytes, keratinocytes, thyroid follicular cells and bone marrow cells. It will be evident that one can utilize any of the gene delivery vehicles noted above for introduction into tissue cells in an *ex vivo* context. Protocols for physical and chemical methods of uptake include calcium phosphate precipitation, direct microinjection of DNA into intact target cells, and electroporation. Other suitable procedures include the use of DNA bound to ligand, DNA linked to an inactivated adenovirus (Cotten et al., *PNAS 89*:6094, 1992), bombardment with DNA bound to particles, liposomes entrapping gene delivery vehicles, and spheroplast fusion whereby *E. coli* containing gene delivery vehicles are stripped of their outer cell walls, and fused to animal cells using polyethylene glycol and viral transduction (Cline et al., *Pharmac. Ther. 29*:69, 1985, and Friedmann et al., *Science 244*:1275, 1989).

Within the context of the present invention, it should be understood that the removed cells may not only be returned to the same animal, but may also be utilized in another, allogeneic, animal. In such a case it is generally preferable to have MHC-matched animals (although not always, *see, e.g.*, Yamamoto et al., "Efficacy of Experimental FIV Vaccines," 1st International Conference of FIV Researchers, University of California at Davis, September 1991).

In addition, it should be understood that a variety of animal cells may be utilized within the context of the present invention, including for example, human, macaque, dog, rat, and mouse cells.

Cells may be removed from a variety of locations including, for example, from a selected tumor. When utilizing tumor cells, the above-described methods may additionally comprise the steps of depleting fibroblasts or other non-contaminating tumor cells subsequent to removing tumor cells from a warm-blooded animal, and/or the step of inactivating the cells, for example, by irradiation. In addition, as noted above, within other embodiments of the invention a gene delivery vehicle may be inserted into non-tumorigenic cells, including for example, cells from the skin (dermal fibroblasts), or from the blood (*e.g.*, peripheral blood leukocytes). If desired, particular fractions of cells such as a T cell subset or stem cells may also be specifically removed from the blood (*see*, for example, PCT WO 91/16116, entitled "Immunoselection Device and Method"). Vector constructs may then be used to modify the removed cells utilizing any of the above-described techniques, followed by return of the cells to the warm-blooded animal.

Within certain preferred embodiments, the gene delivery vehicle is a retrovirus. Retroviruses are RNA viruses with a single positive strand genome which, in general, are nonlytic. Upon infection, the retrovirus reverse transcribes its RNA into DNA, forming a provirus which is inserted into the host cell genome. Preparation of retroviral constructs for use in the present invention is described in greater detail in an application entitled "Recombinant Retroviruses", herein incorporated by reference. The retroviral genome can be divided conceptually into two parts. The "trans-acting" portion consists of the region coding for viral structural proteins, including the group specific antigen (gag) gene for synthesis of the core coat proteins; the pol gene for the synthesis of the reverse transcriptase and integrase enzymes; and the envelope (env) gene for the synthesis of envelope glycoproteins. The "cis-acting" portion consists of regions of the genome that is finally packaged into the viral particle. These regions include the packaging signal, long terminal repeats (LTR) with promoters and polyadenylation sites, and two start sites for DNA replication. The internal or "trans-acting" part of the cloned provirus is replaced by the gene of interest to create a "vector construct". When the vector construct is placed into a cell where viral packaging proteins are present (*see* WO 92/05266), the transcribed RNA will be packaged as a viral particle which , in turn, will bud off from the cell. These particles are used to transduce tissue cells, allowing the vector construct to integrate into the cell genome. Although the vector construct expresses its gene product, the virus carrying it is replication defective because the trans-acting portion of the viral genome is absent. Various assays may be utilized in order to detect the presence of any replication competent infectious retrovirus. One preferred assay is the extended $S^{+}L^{-}$ assay.

Numerous retroviral gene delivery vehicles may be utilized within the context of the present invention, including for example those disclosed within EP 415,731; WO 90/07936; WO 91/0285, WO 94/03622; WO 93/25698; WO 93/25234; U.S. Patent No. 5,219,740; WO 93/11230; WO 93/10218; Vile and Hart, *Cancer Res. 53*:3860-3864, 1993; Vile and Hart, *Cancer Res. 53*:962-967, 1993; Ram et al., *Cancer Res. 53*:83-88, 1993; Takamiya et al., *J. Neurosci. Res. 33*:493-503, 1992; Baba et al., *J. Neurosurg. 79*:729-735, 1993 (U.S. Patent No. 4,777,127, GB 2,200,651, EP 345,242 and WO 91/02805). Preferred retroviral vectors include murine leukemia amphotropic or xenotropic, or VsVg pseudotype vectors (*see*, WO 92/14829, incorporated herein by reference).

Retroviral gene delivery vehicles of the present invention may be readily constructed from a wide variety of retroviruses including, for example, B-, C-, and D-type retroviruses, as well as spumaviruses and lentiviruses (*see*, RNA Tumor Viruses, Second Edition, Cold Spring Harbor Laboratory, 1985). Briefly, viruses are often classified according to their morphology as seen under electron microscopy. Type "B" retroviruses appear to have an eccentric core, while type "C" retroviruses have a central core. Type "D" retroviruses have a morphology intermediate between type B and type C retroviruses. Representative examples of suitable retroviruses include a variety of xenotropic retroviruses (*e.g.*, NZB-X1, NZB-X2, and NZB_{9-1} (*see*, O'Neill et al., *J. Vir. 53*:100-106, 1985)) and polytropic retroviruses (*e.g.*, MCF and MCF-MLV (*see*, Kelly et al., *J. Vir. 45*(1):291-298, 1983)). Such retroviruses may be readily obtained from depositories or collections such as the American Type Culture Collection ("ATCC"; Rockville, Maryland), or isolated from known sources using commonly available techniques.

Particularly preferred retroviruses for the preparation or construction of retroviral gene delivery vehicles of the present invention include retroviruses such as Avian Leukosis Virus, Bovine Leukemia Virus, Murine Leukemia Virus, Mink-Cell Focus-Inducing Virus, Murine Sarcoma Virus, Reticuloendotheliosis Virus, and Rous Sarcoma Virus. Particularly preferred Murine Leukemia Viruses include 4070A and 1504A (Hartley and Rowe, *J. Virol. 19*:19-25, 1976), Abelson (ATCC No. VR-999), Friend (ATCC No. VR-245), Graffi, Gross (ATCC No. VR-590), Kirsten, Harvey Sarcoma Virus and Rauscher (ATCC No. VR-998), and Moloney Murine Leukemia Virus (ATCC No. VR-190). Particularly preferred Rous Sarcoma Viruses include Bratislava, Bryan high titer (*e.g.*, ATCC Nos. VR-334, VR-657, VR-726, VR-659, and VR-728), Bryan standard, Carr-Zilber, Engelbreth-Holm, Harris, Prague (*e.g.*, ATCC Nos. VR-772 and 45033), and Schmidt-Ruppin (*e.g.* ATCC Nos. VR-724, VR-725, VR-354).

Any of the above retroviruses may be readily utilized in order to assemble or construct retroviral gene delivery vehicles given the disclosure provided herein, and standard recombinant techniques (*e.g.*, Sambrook et al, *Molecular Cloning: A Laboratory Manual*, 2d ed., Cold Spring Harbor Laboratory Press, 1989; Kunkle, *PNAS 82*:488, 1985). In addition, within certain embodiments of the invention, portions of the retroviral gene delivery vehicles may be derived from different retroviruses. For example, within one embodiment of the invention, retrovector LTRs may be derived from a Murine Sarcoma Virus, a tRNA binding site from a Rous Sarcoma Virus, a packaging signal from a Murine Leukemia Virus, and an origin of second strand synthesis from an Avian Leukosis Virus.

Within one aspect of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, one or more heterologous sequences, an origin of second strand DNA synthesis and a 3' LTR, wherein the vector construct lacks gag/pol or env coding sequences. Briefly, Long Terminal Repeats ("LTRs") are subdivided into three elements, designated U5, R, and U3. These elements contain a variety of signals which are responsible for the biological activity of a retrovirus, including for example, promoter and enhancer elements which are located within U3. LTRs may be readily identified in the provirus due to their precise duplication at either end of the genome. As utilized herein, a 5' LTR should be understood to include a 5' promoter element and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector. The 3' LTR should be understood to include a polyadenylation signal, and sufficient LTR sequence to allow reverse transcription and integration of the DNA form of the vector.

The tRNA binding site and origin of second strand DNA synthesis are also important for a retrovirus to be biologically active, and may be readily identified by one of skill in the art. For example, retroviral tRNA binds to a tRNA binding site by Watson-Crick base pairing, and is carried with the retrovirus genome into a viral particle. The tRNA is then utilized as a primer for DNA synthesis by reverse transcriptase. The tRNA binding site may be readily identified based upon its location just downstream from the 5' LTR. Similarly, the origin of second strand DNA synthesis is, as its name implies, important for the second strand DNA synthesis of a retrovirus. This region, which is also referred to as the poly-purine tract, is located just upstream of the 3' LTR.

In addition to a 5' and 3' LTR, tRNA binding site, and origin of second strand DNA synthesis, certain preferred retrovector constructs which are provided herein also comprise a packaging signal, as well as one or more heterologous sequences, each of which is discussed in more detail below.

Within ne aspect of the invention, retrovector constructs are provided which lack both *gag/pol* and *env* coding sequences. As utilized herein, the phrase "lacks

gag/pol or *env* coding sequences" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in *gag/pol* or *env* genes, and in particular, within *gag/pol* or *env* expression cassettes that are used to construct packaging cell lines for the retrovector construct.

As an illustration, within one embodiment of the invention construction of retrovector constructs which lack *gag/pol* or *env* sequences may be accomplished by preparing vector constructs which lack an extended packaging signal. As utilized herein, the phrase "extended packaging signal" refers to a sequence of nucleotides beyond the minimum core sequence which is required for packaging, that allows increased viral titer due to enhanced packaging. As an example, for the Murine Leukemia Virus MoMLV, the minimum core packaging signal is encoded by the sequence beginning from the end of the 5' LTR up through the *Pst* I site. The extended packaging signal of MoMLV includes the sequence beyond nucleotide 567 up through the start of the *gag/pol* gene (nucleotide 621), and beyond nucleotide 1560. Thus, within this embodiment retrovector constructs which lack extended packaging signal may be constructed from the MoMLV by deleting or truncating the packaging signal prior to nucleotide 567.

Within other embodiments of the invention, retrovector constructs are provided wherein the packaging signal that extends into, or overlaps with, retroviral *gag/pol* sequence is deleted or truncated. For example, in the representative case of MoMLV, the packaging signal is deleted or truncated prior to the start of the *gag/pol* gene. Within preferred embodiments of the invention, the packaging signal is terminated at nucleotide 570, 575, 580, 585, 590, 595, 600, 610, 615, or 617.

Within other aspects of the invention, retrovector constructs are provided which include a packaging signal that extends beyond the start of the *gag/pol* gene. When such retrovector constructs are utilized, it is preferable to utilize packaging cell lines for the production of recombinant viral particles wherein the 5' terminal end of the *gag/pol* gene in a *gag/pol* expression cassette has been modified to contain codons which are degenerate for *gag*.

Within other aspects of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral nucleic acid sequence upstream of the 5' LTR. As utilized within the context of the present invention, the phrase "does not contain a retroviral nucleic acid sequence upstream of the 5' LTR" should be understood to mean that the retrovector does not contain at least 20, preferably at least 15, more preferably at least 10, and most preferably at least 8 consecutive nucleotides which are found in a retrovirus, and more specifically, in

a retrovirus which is homologous to the retrovector construct. Within a preferred embodiment, the retrovector constructs do not contain a *env* coding sequence upstream of the 5' LTR.

Within a further aspect of the present invention, retrovector constructs are provided comprising a 5' LTR, a tRNA binding site, a packaging signal, an origin of second strand DNA synthesis and a 3' LTR, wherein the retrovector construct does not contain a retroviral packaging signal sequence downstream of the 3' LTR. As utilized herein, the term "packaging signal sequence" should be understood to mean a sequence sufficient to allow packaging of the RNA genome.

Within another preferred embodiment, the gene delivery vehicle is a Sindbis viral vector. Briefly, the Sindbis virus is the prototype member of the alphavirus genus of the togavirus family. The unsegmented genomic RNA (49S RNA) of Sindbis virus is approximately 11,703 nucleotides in length, contains a 5' cap and a 3' polyadenylated tail, and displays positive polarity. Infectious enveloped Sindbis virus is produced by assembly of the viral nucleocapsid proteins onto the viral genomic RNA in the cytoplasm and budding through the cell membrane embedded with viral encoded glycoproteins. Entry of virus into cells is by endocytosis through clatharin coated pits, fusion of the viral membrane with the endosome, release of the nucleocapsid, and uncoating of the viral genome. During viral replication the genomic 49S RNA serves as template for synthesis of the complementary negative strand. This negative strand in turn serves as template for genomic RNA and an internally initiated 26S subgenomic RNA. The Sindbis viral nonstructural proteins are translated from the genomic RNA while structural proteins are translated from the subgenomic 26S RNA. All viral genes are expressed as a polyprotein and processed into individual proteins by post translational proteolytic cleavage. The packaging sequence resides within the nonstructural coding region, therefore only the genomic 49S RNA is packaged into virions.

Several different Sindbis vector systems may be constructed and utilized within the present invention. Representative examples of such systems include those described within U.S. Patent Nos. 5,091,309 and 5,217,879.

Particularly preferred Sindbis vectors for use within the present invention include those which are described within U.S. Serial No. 08/198,450. Briefly, within one embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, and a Sindbis RNA polymerase recognition sequence. Within other embodiments, the viral junction region has been modified such that viral transcription of the subgenomic fragment is reduced. Within

another embodiment, Sindbis vector constructs are provided comprising a 5' sequence which is capable of initiating transcription of a Sindbis virus, a nucleotide sequence encoding Sindbis non-structural proteins, a first viral junction region which has been inactivated such that viral transcription of the subgenomic fragment is prevented, a second viral junction region which has been modified such that viral transcription of the subgenomic fragment is reduced, and a Sindbis RNA polymerase recognition sequence. Within yet another embodiment, Sindbis cDNA vector constructs are provided comprising the above-described vector constructs, in addition to a 5' promoter which is capable of initiating the synthesis of viral RNA from cDNA, and a 3' sequence which controls transcription termination.

In still further embodiments, the vector constructs described above contain no Sindbis structural proteins in the vector constructs the selected heterologous sequence may be located downstream from the viral junction region; in the vector constructs described above having a second viral junction, the selected heterologous sequence may be located downstream from the second viral junction region, where the heterologous sequence is located downstream, the vector construct may comprise a polylinker located between the viral junction region and said heterologous sequence, and preferably the polylinker does not contain a wild-type Sindbis virus restriction endonuclease recognition sequence.

When Sindbis gene delivery vehicles are utilized, the modified cells are fully viable and healthy, and no other viral antigens (which may well be immunodominant) are expressed. This presents a distinct advantage since the antigenic epitopes expressed can be altered by selective cloning of sub-fragments of the gene for the antigen into the recombinant Sindbis virus, leading to responses against immunogenic epitopes which may otherwise be overshadowed by immunodominant epitopes. Such an approach may be extended to the expression of a peptide having multiple epitopes, one or more of the epitopes being derived from different proteins. Further, this aspect of the invention allows efficient stimulation of cytotoxic T lymphocytes (CTL) directed against antigenic epitopes, and peptide fragments of antigens encoded by sub-fragments of genes, through intracellular synthesis and association of these peptide fragments with MHC class I molecules.

The above-described Sindbis vector constructs, as well as numerous similar vector constructs, may be readily prepared essentially as described in U.S. Serial No. 08/198,450, which is incorporated herein by reference in its entirety.

As will be evident to one of ordinary skill in the art given the disclosure provided herein, when utilizing viral gene delivery vehicles, the efficiency of packaging and hence, viral titer, is to some degree dependent upon the size of the sequence to be packaged. Thus, in order to increase the efficiency of packaging and the production

particle virus, additional non-coding sequences may be added to the gene delivery vehicle. Moreover, within certain embodiments of the invention it may be desired to increase or decrease viral titer. This increase or decrease may be accomplished by increasing or decreasing the size of the heterologous sequence, and hence the efficiency of packaging.

Within one embodiment, gene delivery vehicles may be constructed to include a promoter such as SV40 (*see*, Kriegler et al., *Cell 38*:483, 1984), cytomegalovirus ("CMV") (*see*, Boshart et al., *Cell 41*:521-530, 1991), or an internal ribosomal binding site ("IRBS"). Briefly, with respect to IRBS, the upstream untranslated region of the immunoglobulin heavy chain binding protein has been shown to support the internal engagement of a bicistronic message (*see*, Jacejak and Sarnow, *Nature 353*:90-94, 1991). This sequence is small (300 bp), and may readily be incorporated into a retroviral vector or other gene delivery vehicle in order to express multiple genes from a multi-cistronic message whose cistrons begin with this sequence.

Substances/Nucleotide sequences

As briefly noted above, the gene delivery vehicles provided herein direct the expression of a substance capable of preventing, inhibiting, stabilizing or reversing infectious, cancerous, auto-immune or immune diseases. Such diseases include viral infections such as HIV, HBV and HPV, melanomas, autoimmune diseases such as diabetes, graft vs. host disease, multiple sclerosis, rheumatoid arthritis uveitis, Alzheimer's disease and heart disease. In this regard, the present invention is directed, in part, toward methods for stimulating a specific immune response, either humoral or cell-mediated, to an antigen or pathogenic antigen. More specifically, in one aspect, the present invention provides a method for stimulating a specific immune response by using gene delivery vehicles that direct the expression of an antigen or modified form thereof in susceptible target cells capable of initiating such an immune response to the antigen. Expression of the nucleic acid encoded protein may be transient or stable with time. Where an immune response is to be stimulated to a pathogenic antigen, the gene delivery vehicle is preferably designed to express a modified form of the antigen which will stimulate an immune response and which has reduced pathogenicity relative to the native antigen. This immune response is achieved when cells present antigens in the correct manner, *i.e.*, in the context of the MHC class I and/or II molecules, along with various accessory molecules.

Heterologous sequences which may be carried by the gene delivery vehicle include, for example, sequences which encode antigens which stimulate an immune response, and immunomodulatory factors which assist or inhibit an immune response. Within various embodiments of the invention, the gene delivery vehicle may contain two or more heterologous sequences.

In the cases of disease caused by HIV infection, where immunostimulation is desired, the antigen generated from the recombinant retroviral genome may be of a form which will elicit either or both an HLA class I- or class II-restricted immune response. In the case of HIV envelope protein, for example, the antigen is preferably selected fr m gp 160, gp 120, and gp 41, which may be modified to reduce their pathogenicity. In particular, as noted above, the selected antigen is modified to reduce the possibility of syncytia, to avoid expression of epitopes leading to a disease enhancing immune response, to remove immunodominant, but strain-specific epitopes or to present several strain-specific epitopes, and allow a response capable of eliminating cells infected with most or all strains of HIV. The strain-specific epitopes can be further selected to promote the stimulation of an immune response within an animal which is cross-reactive against other strains of HIV. Antigens from other HIV genes or combinations of genes, such as gag, pol, rev, vif, nef, prot, gag/pol, gag/prot, etc., may also provide protection in particular cases.

HIV is only one example. This approach will be effective against many virally linked diseases or cancers where a characteristic antigen (which does not need to be a membrane protein) is expressed, such as in HPV and cervical carcinoma, HTLV-I-induced leukemias, prostate-specific antigen (PSA) and prostate cancer, mutated p53 and colon carcinoma and various cellular genes such as MAGE 1, MAGE 3, MART 1, tyrosinase and melanoma.

Similarly, the hepatitis B virus presents several different antigens, including among others, three HB "S" antigens (HBsAgs), an HBc antigen (HBcAg), an HBe antigen (HBeAg), and an HBx antigen (HBxAg) (*see* WO 93/15207 and Blum et al., "The Molecular Biology of Hepatitis B Virus," *TIG* 5(5):154-158, 1989). Briefly, the HBeAg results from proteolytic cleavage of P22 precore intermediate and is secreted from the cell. HBeAg is found in serum as a 17 kD protein. The HBcAg is a protein of 183 amino acids, and the HBxAg is a protein of 145 to 154 amino acids, depending on subtype.

HBsAg synthesized in animal cells is glycosylated, assembled and secreted into the cell supernatant (Tiollais et al., *Nature 317*:489-495, 1985). Three different env proteins are encoded by the S region of the HBV genome, which contains three translation start codons (Heerman et al., *J. Virol 52*:396-402, 1984; Tiollais et al., *Nature 317*:489-495, 1985). The large, middle, and major env proteins initiate translation at the first, second and third ATG and the synthesis proceeds to the end of the ORF. The $preS_1$, $preS_2$ and the S gene segments of this ORF are located between the first and second ATG, the second and third ATG, and the third ATG and the end of the ORF, respectively. The three segments encode 119, 55 or 226 amino acids, respectively. The $preS_2$ product binds pHSA (Machida et al., *Gastroenterology 86*:910-918, 1984; Michel et al., *Proc. Natl. Acad. Sci. USA 81*:7708-7712, 1985; Persing et al., *Proc. Natl. Acad. Sci. USA*

82:3440-3444, 1985). Since hepatocytes express a receptor for HSA it has been suggested that pHSA may act as an intermediate receptor, binding to middle S protein and to hepatocyte, resulting virus attachment (Michel et al., *Proc. Natl. Acad. Sci. USA 81*:7708-7712, 1985). The major and large env proteins are either non-glycosylated (p24, p39) or are glycosylated at a site within the S region (gp27, gp42). The middle env protein is glycosylated at a site within the pre-S_2 region (gp33) and may also be glycosylated in the S region (gp36).

As will be evident to one of ordinary skill in the art, various immunogenic portions of the above-described antigens may be combined in order to present an immune response when administered by the gene delivery vehicles described herein. For example, due to the large immunological variability that is found in different geographic regions for the S open reading frame of HBV, particular combinations of antigens may be preferred for administration in particular geographic regions. Briefly, epitopes that are found in all human hepatitis B virus S antigen samples are defined as determinant "*a*". Mutually exclusive subtype determinants however have also been identified by two-dimensional double immunodiffusion (Ouchterlony, *Progr. Allergy 5*:1, 1958). These determinants have been designated "*d*" or "*y*" and "*w*" or "*r*" (LeBouvier, *J. Infect. 123*:671, 1971; Bancroft et al., *J. Immunol. 109*:842, 1972; Courouce et al., *Bibl. Haematol. 42*:1-158, 1976). The immunological variability is due to single nucleotide substitutions in two areas of the hepatitis B virus S open reading frame resulting in the following amino acid changes: (1) exchange of lysine-122 to arginine in the hepatitis B virus S open reading frame causes a subtype shift from *d* to *y*, and (2) exchange of arginine-160 to lysine causes the shift from subtype *r* to *w*. In black Africa, subtype *adw*$_2$ is predominant, whereas in the U.S. and northern Europe the subtype *adw*$_2$ is more abundant (*Molecular Biology of the Hepatitis B Virus*, McLachlan (ed.), CRC Press, 1991). As will be evident to one of ordinary skill in the art, it is generally preferred to construct a gene delivery vehicle for administration which is appropriate to the particular hepatitis B virus subtype which is prevalent in the geographical region of administration. Subtypes of a particular region may be determined by two-dimensional double immunodiffusion or, preferably, by sequencing the S open reading frame of HBV virus isolated from individuals within that region.

Also presented by HBV are pol ("HBV pol"), ORF 5, and ORF 6 antigens. Briefly, the polymerase open reading frame of HBV encodes reverse transcriptase activity found in virions and core-like particles in infected liver. The polymerase protein consists of at least two domains: the amino terminal domain encodes the protein that primes reverse transcription, and the carboxyl terminal domain which encodes reverse transcriptase and RNase H activity. Immunogenic portions of HBV pol may be determined utilizing methods known in the art, and administered using the gene delivery vehicle in order to generate an

immune response within a warm-blooded animal. Similarly, other HBV antigens such as ORF 5 and ORF 6, (Miller et al., *Hepatology 9*:322-327, 1989), may be expressed utilizing gene delivery vehicles as described herein.

Molecularly cloned genomes which encode the substances described herein may be obtained from a variety of sources including, for example, the American Type Culture Collection (ATCC, Rockville, Maryland). For example, ATCC No. 45020 contains the total genomic DNA of hepatitis B (extracted from purified Dane particles) (*see*, Figure 3 of Blum et al., *TIG 5*(5):154-158, 1989) in the Bam HI site of pBR322 (Moriarty et al., *Proc. Natl. Acad. Sci. USA 78*:2606-2610, 1981).

As noted above, within another aspect of the present invention, a gene delivery vehicle is provided which directs the expression of a non-tumorigenic, altered cellular component, such as a ras (ras*) gene (*see* WO 93/10814). Briefly, the ras* gene is an attractive target because it is causally linked to the neoplastic phenotype, and indeed may be necessary for the induction and maintenance of tumorigenesis in a wide variety of distinct cancers, such as pancreatic carcinoma, colon carcinoma and lung adenocarcinoma. In addition, ras* genes are found in pre-neoplastic tumors, and therefore immune intervention therapy may be applied prior to detection of a malignant tumor.

Normal ras genes are non-tumorigenic and ubiquitous in all mammals. They are highly conserved in evolution and appear to play an important role in maintenance of the cell cycle and normal growth properties. The normal ras protein is a G-protein which binds GTP and has GTPase activity, and is involved in transmitting signals from the external milieu to the inside of the cell, thereby allowing a cell to respond to its environment. Ras* genes on the other hand alter the normal growth regulation of neoplastic cells by uncoupling cellular behavior from the environment, thus leading to the uncontrolled proliferation of neoplastic cells. Mutation of the ras gene is believed to be an early event in carcinogenesis (Kumar et al., "Activation of ras Oncogenes Preceding the Onset of Neoplasia," *Science 248*:1101-1104, 1990), which, if treated early, may prevent tumorigenesis.

Ras* genes occur in a wide variety of cancers, including for example, pancreatic, colon, and lung adenocarcinomas (*see* Table 1 below).

TABLE 1

Tumor type	Incidence of ras mutations
Pancreatic Adenocarcinoma	90%
Colon Adenoma	50%
Colon Adenocarcinoma	50%
Seminoma	40%
Lung Adenocarcinoma	30%
Myelodisplatic Syndrome	30%
Acute Myelogenous leukemia	30%
Keratinoacanthoma	30%
Thyroid carcinoma	25%
Melanomas	20%
Bladder carcinoma	6%

The spectrum of mutations occurring in the ras* genes found in a variety of cancers is quite limited. These mutations alter the GTPase activity of the ras protein by converting the normal on/off switch to a constitutive ON position. Tumorigenic mutations in ras* occur primarily (*in vivo*) in only 3 codons: 12, 13 and 61. Codon 12 mutations are the most prevalent in both human and animal tumors. Table 2 below sets forth the incidence of mutations at codons 12 and 13 for various human tumors.

TABLE 2

Approximate percentage of specific mutations at codons 12 and 13 of ras*

Tumor type/Mutation	GAT Asp	GAC Asp	AGT Ser	CGT Arg	TGT Cys	GTT Val	GCT Ala
Pancreatic Carcinoma	47%	2%	2%	10%	12%	27%	<1%
Colorectal Adenoma or Carcinoma	39%	23%	3%	<1%	9%	23%	2%
Lung Carcinoma	17%	4%	4%	4%	40%	30%	<1%

Table 3 summarizes known *in vivo* mutations (codons 12, 13 and 61) which activate human ras, as well as potential mutations which have *in vitro* transforming activity. Potential mutations with *in vitro* transforming activity were produced by the systematic substitution of amino acids for the normal codon (*e.g.*, other amino acids were substituted for the normal glycine at position 12). Such mutations, while not presently known to occur

in humans or animals, may serve as the basis for an anti-cancer immunotherapeutic if they are eventually found to arise *in vivo*.

Table 3

Amino acid substitutions that activate human *ras* proteins

Amino Acid Mutant Codon	Gly 12	Gly 13	Ala 59	Gln 61	Glu 63	Asn 116	Lys 117	Asp 119
In vivo	Val	Asp		Arg				
	Arg	Val		His				
	Asp	Arg		Leu				
	Cys							
	Ala							
	Ser							
	Phe							
In vitro	Ala	Ser	Thr	Val	Lys	His	Glu	His
	Asn			Ala		Ile	Arg	Glu
	Gln			Cys				Ala
	Glu			Asn				Asn
	His			Ile				
	Ile			Met				
	Leu			Thr				
	Lys			Tyr				
	Met			Trp				
	Phe			Phe				
	Ser			Gly				
	Thr							
	Trp							
	Tyr							

Alterations as described above result in the production of proteins containing novel coding sequence(s). The novel proteins encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequences (ras*).

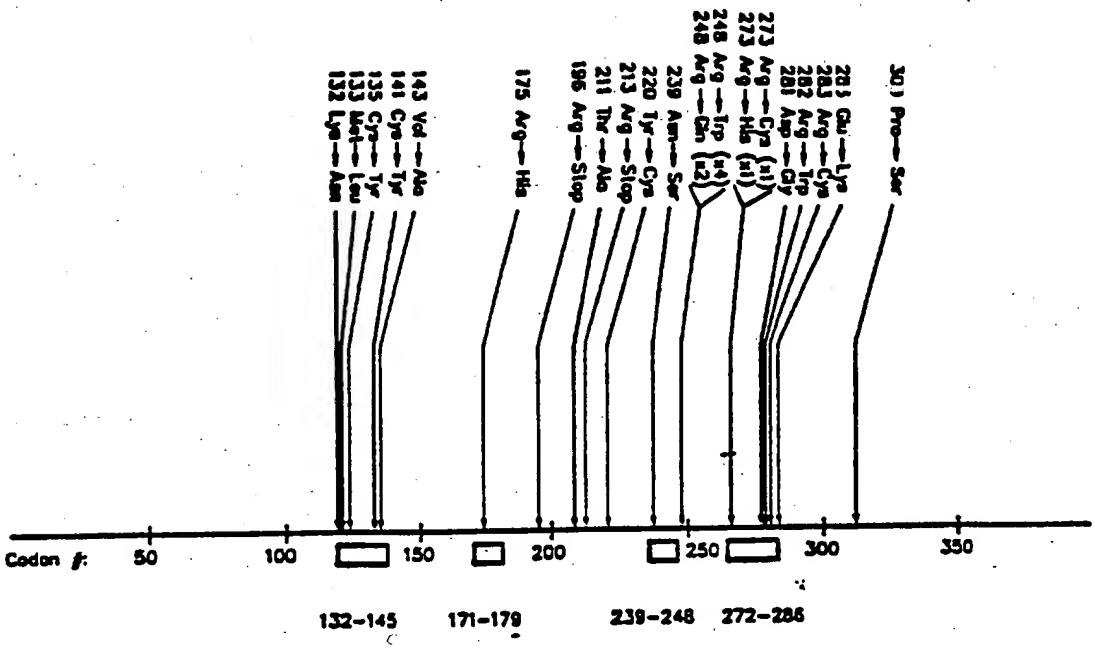
Within another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of an altered p53 (p53*) gene. Briefly, p53 is a nuclear phosphoprotein which was originally discovered in extracts of transformed cells, and thus was initially classified as an oncogene (Linzer and Levine, *Cell 17*:43-52, 1979; Lane and Crawford, *Nature 278*:261-263, 1979). It was later discovered that the original p53 cDNA clones were mutant forms of p53 (Hinds et al., *J. Virol. 63*:739-746, 1989). It now appears that p53 is a tumor suppressor gene, which negatively

regulates the cell cycle, and that mutation of this gene may lead to tumor formation. Of colon carcinomas that have been studied, 75%-80% show a loss of both p53 alleles, one through deletion, and the other through point mutation. Similar mutations are found in lung cancer, and in brain and breast tumors.

The majority of p53 mutations (*e.g.*, $p53^{*1}$, $p53^{*2}$, etc.) are clustered between amino-acid residues 130 to 290 (*see* Levine et al., *Nature 351*:453-456, 1991; *see also* the following references which describe specific mutations in more detail: Baker et al., *Science 244*:217-221, 1989; Nigro et al., *Nature 342*:705-708, 1989 (p53 mutations cluster at four "hot spots" which coincide with the four highly conserved regions of the genes and these mutations are observed in human brain, breast, lung and colon tumors); Vogelstein, *Nature 348*:681-682, 1990; Takahashi et al., *Science 246*:491-494, 1989; Iggo et al., *Lancet 335*:675-679, 1990; James et al., *Proc. Natl. Acad. Sci. USA 86*:2858-2862, 1989; Mackay et al., *Lancet 11*:1384-1385,1988; Kelman et al., *Blood 74*:2318-2324, 1989; Malkin et al., *Science 250*:1233-1238, 1990; Baker et al., *Cancer Res. 50*:7717-7722, 1991; Chiba et al., *Oncogene 5*:1603-1610, 1990 (pathogenesis of early stage non-small cell lung cancer is associated with somatic mutations in the p53 gene between codons 132 to 283); Prosser et al., *Oncogene 5*:1573-1579, 1990 (mutations in the p53 gene coding for amino acids 126 through 224 were identified in primary breast cancer); Cheng and Hass, *Mol. Cell. Biol. 10*:5502-5509, 1990; Bartek et al., *Oncogene 5*:893-899, 1990; Rodrigues et al., *Proc. Natl. Acad. Sci. USA 87*:7555-7559, 1990; Menon et al., *Proc. Natl. Acad. Sci. USA 87*:5435-5439, 1990; Mulligan et al., *Proc. Natl. Acad. Sci. USA 87*:5863-5867, 1990; and Romano et al., *Oncogene 4*:1483-1488, 1990 (identification of a p53 mutation at codon 156 in human osteosarcoma derived cell line HOS-SL)).

Certain alterations of the p53 gene may be due to certain specific toxins. For example, Bressac et al. (*Nature 350*:429-431, 1991) describes specific G to T mutations in codon 249, in patients affected with hepatocellular carcinoma. One suggested causative agent of this mutation is aflatoxin B_1, a liver carcinogen which is known to be a food contaminant in Africa.

Four regions of the gene that are particularly affected occur at residues 132-145, 171-179, 239-248, and 272-286:



Three "hot spots" of particular interest occur at residues 175, 248 and 273 (Levine et al., *Nature 351*:453-456, 1991). These alterations as well as others which are described above result in the production of protein(s) which contain novel coding sequence(s). The novel proteins encoded by these sequences may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding regions may be utilized to destroy tumorigenic cells containing the altered sequence ($p53^*$).

Within yet another embodiment of the present invention, a gene delivery vehicle is provided which directs the expression of an altered Rb (Rb^*) gene. Briefly, retinoblastoma is a childhood eye cancer associated with the loss of a gene locus designated Rb, which is located in chromosome band 13q14. A gene from this region has been cloned which produces a nuclear phosphoprotein of about 110kd (Friend et al., *Nature 323*:643, 1986; Lee et al., *Science 235*:1394, 1987; and Fung et al., *Science 236*:1657, 1987).

Rb is believed to be a negative regulator of cellular proliferation, and has a role in transcriptional control and cell-cycle regulation. Rb binds to at least seven proteins found in the nucleus, and in particular, appears to be involved with a cellular transcription factor which has been designated both E2F (Bagchi et al., *Cell 62*:659-669, 1990) and DRTF (Shivji and La Thangue, *Mol. Cell. Biol. 11*:1686-1695, 1991). Rb is believed to restrict cellular growth by sequestering a variety f nuclear proteins involved in cellular proliferation.

Deletions within the Rb gene have been detected which evidence that the Rb gene may be responsible for tumorigenicity. These deletions include, for example, a

deletion in exon 21 in a prostate cancer and bladder cancer cell line (Bookstein et al., *Science 247*:712-715, 1990; Horowitz et al., *Science 243*:937, 1989), a deletion of exon 16 in a small-cell carcinoma of the lung (Shew et al., *Cell Growth and Diff. 1*:17. 1990), and a deletion between exons 21 and 27 (Shew et al., *Proc. Natl. Acad. Sci. USA 87*:6, 1990). Deletion of these exons results in the production of a protein containing a novel coding sequence at the junction of the deleted exons. This novel protein coding sequence may be used as a marker of tumorigenic cells, and an immune response directed against this novel coding region may eliminate tumorigenic cells containing the Rb exon deletion.

Within another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of an altered gene which causes Wilms' tumor. Briefly, Wilms' tumor is typically found in children younger than 16 years of age. One child in 10,000 will develop this tumor, which comprises approximately 5% of childhood cancers. The tumor usually presents itself as a large abdominal mass which is surrounded by a fibrous pseudocapsule. Approximately 7% of the tumors are multifocal in one kidney, and 5.4% are involved with both kidneys. The Wilms' tumor gene has been localized to chromosome 11p13, and a cDNA clone (wt1) has been isolated that is characteristic of a tumor suppressor gene (Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; and Haber et al., *Cell 61*:1257, 1990). The wt1 gene encodes a protein which contains four zinc fingers and a glutamine and proline rich amino terminus. Such structures are believed to be associated with transcriptional and regulatory functions.

Mutations of the Wilms' tumor gene include the insertion of lysine, threonine and serine between the third and forth zinc fingers. A wt1 protein which contains such insertions does not bind to the EGR-1 site. A second alternative mutation results in the insertion of about 17 amino acids in the region immediately NH_2-terminal to the zinc finger domain (Madden et al., *Science 253*:1550-1553, 1991; Call et al., *Cell 60*:509, 1990; Gessler et al., *Nature 343*:744, 1990; Rose et al., *Cell 60*:495, 1990; Haber et al., *Cell 61*:1257, 1990; and Buckler et al., *Mol. Cell. Biol. 11*:1707, 1991).

Alterations as described above result in the production of protein(s) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s), which cause Wilms' tumor.

Within yet another embodiment of this aspect of the present invention, a gene delivery vehicle is pr vided which directs the expression of an altered mucin. Mucins are large molecular weight glycoproteins which contain approximately 50% carbohydrate. Polymorphic epithelial mucin (PEM) is a tumor-associated mucin (Girling et al., *Int. J.*

Cancer 43:1072-1076, 1989) which is found in the serum of cancer patients. The full-length cDNA sequence has been identified (Gendler et al., *J. Biol. Chem. 265*(25):15286-15293, 1990; Lan et al., *J. Biol. Chem. 265*(25):15294-15299, 1990; and Ligtenberg et al., *J. Biol. Chem. 265*:5573-5578, 1990). Breast tumors and pancreatic tumors both express a mucin with an identical core sequence, containing a 20 amino-acid tandem repeat (Jerome et al., *Cancer Res. 51*:2908-2916, 1991). CTL lines which have been developed to breast tumors which cross-react with pancreatic tumor targets, and further appear to specifically recognize the specific 20 amino-acid tandem repeat (Jerome et al., *supra*). A sequence encoding one or more of the 20 amino-acid tandem repeats may be expressed by a gene delivery vehicle of the present invention, in order to develop an immune response against tumor cells which contain this sequence.

Within another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of an altered DCC (deleted in colorectal carcinomas) gene. Briefly, a very common region of allelic loss in colorectal tumors is chromosome 18q, which is lost in more than 70% of carcinomas, and in almost 50% of late adenomas. A presumptive tumor suppressor gene (DCC) from this region has been identified (Fearon et al., 1990), which encodes a protein with significant homology to cell-surface adhesion molecules, such as neural cell-adhesion molecule (NCAM) and contactin (reviewed by Edelman in *Biochem 27*:3533-3543, 1988). This protein is believed to play a role in the development of colorectal tumors, perhaps through alterations in normal cell-cell and/or cell-extracellular matrix interactions.

The DCC gene is expressed in normal colonic mucosa, but its expression is reduced or absent in the majority of colorectal carcinomas (Solomon, *Nature 343*:412-414, 1990). This loss of expression has been associated in some cases with somatic mutations of the DCC gene. A contiguous stretch of DNA comprising 370kb has been cloned which encodes an approximately 750 amino acid protein (Fearon et al., "Identification of a Chromosome 18q Gene That Is Altered in Colorectal Cancers," *Science 247*:49-56, 1990).

Within yet another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of MCC or APC. Both MCC (mutated in colorectal cancer) and APC have been identified as tumor suppressor genes (Kinzler et al., *Science 251*:1366-1370, 1991) which undergo mutation in familial adenomatous polyposis (FAP). FAP is believed to be the most common autosomal dominant disease which leads to cancer, and it affects at least 1 in 5,000 individuals in the United States (Nishiho et al., *Science 253*:665-669, 1991). Affected individuals usually develop hundreds to thousands of adenomatous polyps of the colon and rectum, which may progress to carcinoma. Gardner's syndrome ("GS," a variant of FAP) presents desmoid tumors, osteomas, and other neoplasms together with multiple adenomas of the colon and

rectum. This pr liferation is believed to be induced by loss or inactivation of the familial adenomatous polyposis gene (and in particular, MCC and APC) which is found on chromosome 5q.

For example, in Nishiho et al. (*supra*), the following germ line mutations of the APC gene were found in FAP and GS patients: (1) Codon 280, a serine to stop mutation (in a patient with mandibular osteoma), (2) codon 302, an arginine to stop mutation in two separate patients, one with a desmoid tumor, (3) codon 414, an arginine to cysteine mutation in a patient with mandibular osteoma, and (5) codon 713, a serine to stop mutation in another patient with mandibular osteoma (Nishiho et al., *Science 253*:665-669, 1991). In addition, six point mutations were identified in MCC codon numbers 12, 145, 267, 490, 506, and 698, as well as an additional 4 somatic mutations in APC (codons number 289, 332, 438, and 1338).

Alterations as described above result in the production of protein(s) containing novel coding sequence(s). The novel protein(s) encoded by these sequence(s) may be used as a marker of tumorigenic cells, and an immune response directed against these novel coding region(s) may be utilized to destroy tumorigenic cells containing the altered sequence(s) or gene(s) which cause DCC, APC, or MCC.

Within another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of altered ubiquitin. Briefly, ubiquitin is a cellular protein which is involved in cell-cycle control and DNA replication. Other functions of ubiquitin include intracellular protein degradation, heat-shock response, transcriptional regulation, cell-cycle control, and cell-cell interaction. Ubiquitin is believed to be a marker molecule that targets proteins for a variety of metabolic fates, and a cDNA sequence which encodes this protein has been identified (Lund et al., "Nucleotide sequence analysis of a cDNA encoding human ubiquitin reveals that ubiquitin is synthesized as a precursor," *J. Biol. Chem. 263*:4926-4931, 1985).

A mutant ubiquitin (ubiquitin*) has recently been identified in a human colon carcinoma cell line (Mafune et al., *Arch.-Surg. 126*:462-466, 1991). This tumor cell contains a novel fusion protein consisting of a hybrid ubiquitin-ribosomal protein S27a. The fusion junction of this protein results in a novel nonself protein sequence which may be immunogenic, and therefore used to eliminate tumor cells carrying this fusion protein.

Within yet another embodiment of this aspect of the present invention, a gene delivery vehicle is provided which directs the expression of altered bcr/abl. Briefly, in tumor cells from almost all patients with chronic myelogenous leukemia, the Philadelphia chromosome, a fusion of chromosomes 9 and 22, directs the synthesis of the fused $P210^{bcr/abl}$ protein. This hybrid gene encodes a 210kD phosphoprotein with disregulated protein-kinase activity which leads to the chronic myel genous leukemia (Daley et al.,

Science 247:824-829, 1990; Shtivelman et al., *Nature 315*:550-554, 1985; Ben-Neriah et al., *Science 233*:212-214, 1986; and Shtivelman et al., *Cell 47*:277-284, 1986). The fusion junction of these two chromosomes results in a novel nonself protein sequence which may be immunogenic, and thus used to eliminate tumor cells carrying this fusion protein.

Within another embodiment of this aspect of the invention, gene delivery vehicles are provided which direct the expression of a substance such as MAGE 1, MAGE 3, MART-1, tyrosine hydroxylase or a protein that has been considered "normal," but is associated with, or an immune response (especially a cellular immune response) against the protein is associated with, the presence of tumors in a patient.

As noted above, at least one immunogenic portion of a substance may be incorporated into a gene delivery vehicle. The immunogenic portion(s) which are incorporated into the gene delivery vehicle may be of varying length, although it is generally preferred that the portions be at least 8 amino acids long, and may include an entire protein. Immunogenicity of a particular sequence is often difficult to predict. However, peptide epitopes may be predicted utilizing class I peptide binding motifs, such as the motif described by Falk et al. for HLA A2.1 (*Nature 351*:290, 1991). From this analysis, peptides may be synthesized and used as targets in an *in vitro* cytotoxic assay. Other assays, however, may also be utilized, including, for example, ELISA which detects the presence of antibodies against the newly introduced vector-encoded proteins, as well as assays which test for T helper cells, such as gamma-interferon assays, IL-2 production assays, and proliferation assays.

Immunogenic portions may also be selected by other methods. For example, the HLA A2.1/K^b transgenic mouse and the HLA-B27 transgenic mouse have been shown to be useful as models for human T-cell recognition of viral antigens. Briefly, in the influenza and hepatitis B viral systems, the murine T-cell receptor repertoire recognizes the same antigenic determinants recognized by human T-cells. In both systems, the CTL response generated in the HLA A2.1/K^b transgenic mouse is directed toward virtually the same epitope as those recognized by human CTLs of the HLA A2.1 haplotype (Vitiello et al., *J. Exp. Med. 173*:1007-1015, 1991; Vitiello et al., *Abstract of Molecular Biology of Hepatitis B Virus Symposia*, 1992). Particularly preferred immunogenic portions for incorporation into vector constructs include HBeAg, HBcAg, and HBsAgs.

Additional immunogenic portions of the hepatitis B virus may be obtained by truncating the coding sequence at various locations including, for example, the following sites: Bst UI, Ssp I, Ppu M1, and Msp I (Valenzuela et al., *Nature 280*:815-19, 1979; Valenzuela et al., *Animal Virus Genetics: ICN/UCLA Symp. Mol. Cell Biol.*, 1980, B. N. Fields and R. Jaenisch (eds.), pp. 57-70, New York: Academic).

The methods described herein may also be applied to the treatment of hepatitis C infections. The genomic RNA of HCV has recently been determined to have a sequence of 9379 nucleotides (Choq et al., *Proc. Natl. Acad. Sci. USA 88*:2451-2455, 1991; Choo et al., *Brit. Med. Bull. 46*(2):423-441, 1990; Okamoto et al., *J. Gen. Vir. 72*:2697-2704, 1991; *see also* Genbank Accession No. M67463, Intelligenetics (Mountain View, California). This sequence expresses a polyprotein precursor of 3011 amino acids, which has significant homology to proteins of the flavivirus family. The polyprotein precursor is cleaved to yield several different viral proteins, including C (nucleocapsid protein) E1, E2/NS1, and non-structural proteins NS2, NS3, NS4, and NS5 (Houghton et al., *Hepatology 14*:381-388, 1991). Preferred immunogenic portion(s) of hepatitis C may be found in the C and NS3-NS4 regions since these regions are the most conserved among various types of hepatitis C virus (Houghton et al., *Hepatology 14*:381-388, 1991). Particularly preferred immunogenic portions may be determined by a variety of methods. For example, as noted above for the hepatitis B virus, identification of immunogenic portions of the polyprotein may be predicted based upon amino acid sequence. Briefly, various computer programs which are known to those of ordinary skill in the art may be utilized to predict class I binding peptides. For example, peptide epitopes which can bind HLA A2.1 molecules may be predicted utilizing the HLA A2.1 motif described by Falk et al. (*Nature 351*:290, 1991). From this analysis, peptides are synthesized and used as targets in an *in vitro* cytotoxic assay.

Within another aspect of the present invention, methods are provided for destroying hepatitis B carcinoma cells comprising the step of administering to a warm-blooded animal a gene delivery vehicle which directs the expression of an immunogenic portion of antigen X, such that an immune response is generated. Sequences which encode the HBxAg may readily be obtained by one of skill in the art given the disclosure provided herein. Briefly, within one embodiment of the present invention, a 642 bp Nco I-Taq I is recovered from ATCC 45020, and inserted into gene delivery vehicles as described above for other hepatitis B antigens.

The X antigen, however, is a known transactivator which may function in a manner similar to other potential oncogenes (*e.g.*, E1A). Thus, it is generally preferable to first alter the X antigen such that the gene product is non-tumorigenic before inserting it into a gene delivery vehicle. Various methods may be utilized to render the X antigen non-tumorigenic including, for example, by truncation, point mutation, addition of premature stop codons, r phosphorylation site alteration. Within one embodiment, the sequence or gene of interest which encodes the X antigen is truncated. Truncation may produce a variety of fragments, although it is generally preferable to retain greater than or equal to 50% of the encoding gene sequence. In addition, it is necessary that any truncation leave

intact some of the immunogenic sequences of the gene product. Alternatively, within another embodiment, multiple translational termination codons may be introduced into the gene. Insertion of termination codons prematurely terminates protein expression, thus preventing expression of the transforming portion of the protein.

The X gene or modified versions thereof may be tested for tumorigenicity in a variety of ways. Representative assays include tumor formation in nude mice, colony formation in soft agar, and preparation of transgenic animals, such as transgenic mice.

Within another aspect of the present invention, gene delivery vehicles may be prepared which direct the co-expression of several of the above-described immunogenic portions (as well as immunomodulatory factors, if desired). For example, within one embodiment gene delivery vehicles may be prepared which direct the co-expression of both an immunogenic portion of the hepatitis B antigen, as well as an immunogenic portion of the hepatitis C polyprotein. Such vehicles may be administered as described above and below, in order to prevent or treat acute and chronic hepatitis infections of either type B or C. Similarly, within other embodiments gene delivery vehicles may be prepared which direct the co-expression of both an immunogenic portion of the hepatitis B X antigen, as well as an immunogenic portion of the hepatitis C polyprotein. Such vehicles may similarly be administered in order to treat hepatocellular carcinoma which is associated with either hepatitis B or C. In addition, because those individuals chronically infected with hepatitis B and C are at higher risk for developing hepatocellular carcinoma, such a vector may also be utilized as a prophylactic treatment for the disease.

As noted above, more than one immunogenic portion may be incorporated into the gene delivery vehicle. For example, a gene delivery vehicle may express (either separately or as one construct) all or immunogenic portions of HBcAg, HBeAg, HBsAgs, HBxAg, as well as immunogenic portions of HCV antigens as discussed below. In addition, as noted above, the gene delivery vehicle may also co-express an immunomodulatory factor, such as alpha interferon (Finter et al., *Drugs 42*(5):749-765, 1991; U.S. Patent No. 4,892,743; U.S. Patent No. 4,966,843; WO 85/02862; Nagata et al., Nature 284:316-320, 1980; Familletti et al., *Methods in Enz. 78*:387-394, 1981; Twu et al., *Proc. Natl. Acad. Sci. USA 86*:2046-2050, 1989; Faktor et al., *Oncogene 5*:867-872, 1990), beta interferon (Seif et al., *J. Virol. 65*:664-671, 1991), gamma interferons (Radford et al., *The American Society of Hepatology* 2008-2015, 1991; Watanabe et al., *PNAS 86*:9456-9460, 1989; Gansbacher et al., *Cancer Research 50*:7820-7825, 1990; Maio et al., *Can. Immunol. Immunother. 30*:34-42, 1989; U.S. Patent No. 4,762,791; U.S. Patent No. 4,727,138), G-CSF (U.S. Patent Nos. 4,999,291 and 4,810,643), GM-CSF (WO 85/04188), TNFs (Jayaraman et al., *J. Immunology 144*:942-951, 1990), Interleukin-2 (IL-2) (Karupiah et al., *J. Immunology 144*:290-298, 1990; Weber et al., *J.*

Exp. Med. 166:1716-1733, 1987; Gansbacher et al., *J. Exp. Med. 172*:1217-1224, 1990; U.S. Patent No. 4,738,927), IL-4 (Tepper et al., *Cell 57*:503-512, 1989; Golumbek et al., *Science 254*:713-716, 1991; U.S. Patent No. 5,017,691), IL-5 (Sanderson et al., *J. Exp. Med. 162*:60-74, 1985; Lopez et al., *Cell. Imm. Today 13*:495-500, 1992), IL-6 (Brakenhof et al., *J. Immunol. 139*:4116-4121, 1987; WO 90/06370), IL-12 (Stern et al., *Proc. Natl. Acad. Sci. USA 87*:6808-6812, 1990; Gately et al., *J. Immunol. 147*:874-882, 1991), ICAM-1 (Altman et al., *Nature 338*:512-514, 1989), ICAM-2, LFA-1, LFA-3, MHC class I molecules, MHC class II molecules, b_2-microglobulin, chaperones, CD3, B7.1-.3 (Freedman et al., *J. Immonol. 137*:3260, 1987), MHC-linked transporter proteins or analogs thereof (Powis et al., *Nature 354*:528-531, 1991).

The choice of which immunomodulatory factor to include within a gene delivery vehicle may be based upon known therapeutic effects of the factor, known defects in the target antigen presenting cell, or experimentally determined.

As noted above, sequences which encode the above-described antigens, proteins or factors may be readily obtained from a variety of sources, including for example depositories such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as British Bio-Technology Limited (Cowley, Oxford England). Representative examples include BBG 12 (containing the GM-CSF gene coding for the mature protein of 127 amino acids), BBG 6 (which contains sequences encoding gamma interferon), ATCC No. 39656 (which contains sequences encoding TNF), ATCC No. 20663 (which contains sequences encoding alpha interferon), ATCC Nos. 31902, 31902, and 39517 (which contain sequences encoding beta interferon), ATCC No. 67024 (which contains a sequence which encodes Interleukin-1b), ATCC Nos. 39405, 39452, 39516, 39626, and 39673 (which contain sequences encoding Interleukin-2), ATCC Nos. 59399, 59398, and 67326 (which contain sequences encoding Interleukin-3), ATCC No. 57592 (which contains sequences encoding Interleukin-4), ATCC Nos. 59394 and 59395 (which contain sequences encoding Interleukin-5), and ATCC No. 67153 (which contains sequences encoding Interleukin-6).

Sequences which encode the above-described feline leukemia virus and feline immunodeficiency virus antigens may be prepared as described within the references cited above, or obtained from a variety of sources. For example, sequences which encode the envelope protein of FeLV may be readily obtained from the American Type Culture Collection ("ATCC"; Rockville, Maryland) (*see, for example,* ATCC Nos. 39528, 39529, and 39530). Similarly, the AIDS Repository (Division f AIDS, National Institute of Allergy and Infectious Disease, Bethesda, Maryland; *see* NIH Publication No. N2-1536) holds a deposit of a plasmid clone which contains a sequence encoding a full-length, replication competent FeLV (*e.g.*, clone p61E-FeLV, Catalog No. 109), as well as a

deposit of a plasmid clone which contains a sequence encoding a feline immunodeficiency virus (*e.g.*, clone pFIV-14-Petaluma, Catalog No. 851).

Sequences which encode the above-described altered cellular components may be obtained from a variety of sources. For example, plasmids which contain sequences that encode altered cellular products may be obtained from a depository such as the American Type Culture Collection (ATCC, Rockville, Maryland), or from commercial sources such as Advanced Biotechnologies (Columbia, Maryland). Representative examples of plasmids containing some of the above-described sequences include ATCC No. 41000 (containing a G to T mutation in the 12th codon of ras), and ATCC No. 41049 (containing a G to A mutation in the 12th codon).

Alternatively, plasmids which encode normal cellular components may also be obtained from depositories such as the ATCC (*see, for example*, ATCC No. 41001 which contains a sequence which encodes the normal ras protein, ATCC No. 57103 which encodes abl; and ATCC Nos. 59120 or 59121 which encode the bcr locus) and mutated to form the altered cellular component. Methods for mutagenizing particular sites may readily be accomplished using methods known in the art (*see* Sambrook et al., *supra.*, 15.3 *et seq.*). In particular, point mutations of normal cellular components such as ras may readily be accomplished by site-directed mutagenesis of the particular codon, for example, codons 12, 13 or 61.

Alternatively, cDNA sequences which encode the above heterologous sequences may be obtained from cells which express or contain the sequences. Briefly, within one embodiment mRNA from a cell which expresses the gene of interest is reverse transcribed with reverse transcriptase using oligo dT or random primers. The single stranded cDNA may then be amplified by PCR (*see*, U.S. Patent Nos. 4,683,202, 4,683,195, and 4,800,159. *See also, PCR Technology: Principles and Applications for DNA Amplification*, Erlich (ed.), Stockton Press, 1989) utilizing oligonucleotide primers complementary to sequences on either side of desired sequences. In particular, a double stranded DNA is denatured by heating in the presence of heat stable Taq polymerase, sequence specific DNA primers, ATP, CTP, GTP and TTP. Double-stranded DNA is produced when synthesis is complete. This cycle may be repeated many times, resulting in a factorial amplification of the desired DNA.

Sequences which encode the above-described proteins may also be synthesized, for example, on an Applied Biosystems Inc. DNA synthesizer (*e.g.*, APB DNA synthesizer model 392 (Foster City, California).

Routes of Administration

As noted above, various methods may be utilized to administer gene delivery vehicles of the present invention, including nucleic acids which encode the immunogenic portion(s) discussed above, to warm-blooded animals such as humans, directly. Suitable methods include, for example, various physical methods such as direct DNA injection (Acsadi et al., *Nature 352*:815-818, 1991), and microprojectile bombardment (Williams et al., *PNAS 88*:2726-2730, 1991).

Within an *in vivo* context, the gene delivery vehicle can be injected into the interstitial space of tissues including muscle, brain, liver, skin, spleen or blood (*see*, WO 90/11092). Administration may also be accomplished by intravenous injection or direct catheter infusion into the cavities of the body (*see*, WO 93/00051), discussed in more detail below.

It is generally preferred that administration of the gene delivery vehicles at multiple sites be via at least two injections. In this regard, suitable modes of administration include intramuscular, intradermal and subcutaneous injections, with at least one of the injections preferably being intramuscular. In particularly preferred embodiments, two or more of the injections are intramuscular. However, although administration via injections is preferred, it will be evident that the gene delivery vehicles may be administered through multiple topical or separate ocular administrations. Further, a number of additional routes are suitable for use within the present invention when combined with one or more of the routes briefly noted above, including intraperitoneal, intracranial, oral, rectal, nasal, vaginal and sublingual administration. Methods of formulating and administering the gene delivery vehicles at multiple sites through such routes would be evident to those skilled in the art.

Transdermal or topical application of a gene delivery vehicle may be used as a route of administration because the skin is the most expansive and readily accessible organ of the human body. Transdermal delivery systems (TDS) are capable of delivering a drug through intact skin so that it reaches the systemic circulation in sufficient quantity to be therapeutically beneficial. TDS provides a variety of advantages, including elimination of gastrointestinal absorption problems and hepatic first pass effect, reduction of dosage and dose intervals, and improved patient compliance. The major components of TDS are a controlled release device composed of polymers, the drug to be administered, excipients, and enhancers, and a fastening system to fix the device to the skin. A number of polymers have been described which include gelatin, gum arabic, paraffin waxes, and cellulose acetate phthalate (Sogibayasi et al., *J. Controlled Release 29*:177-185, 1994). These polymers can be dermatologically formulated into aqueous, powder or oil phases. The

various combinations can produce lotions, pastes, ointments, creams, and gels, with the aid of emulsifiers.

Topical administration of gene delivery vehicles as described herein may be accomplished by encapsulation in liposomes. Hyaluronic acid has been used as a bioadhesive ligand for the formation of liposomes to enhance adherence and retention to the extracellular matrix in cases of burns and wound healing (Yerushalmi et al., *Arch. Biochem. and Biophys. 313*:267-273, 1994). Methods of liposome preparation can be tailored to control size and morphology. Drug molecules can be either incorporated in the aqueous space or intercalated into the lipid bilayer of liposomes, depending upon the physiochemical characteristics of the drug.

Additionally, ionophoresis may be used to cause increased penetration of ionized substances into or through the skin by the application of an electrical field. This method has the advantage of being able to deliver the drug in a pulsatile manner (Singh et al., *Dermatology 187*:235-238, 1993).

Ocular administration of a gene delivery vehicle may also be utilized. The avascular components of the eye allow for topical therapy to be most effective for a number of disease states such as glaucoma, inflammation, and herpes keratitis (Wearley, *Crit. Rev. in Therap. Drug Car. Sys. 8*:331-394, 1991). Systemic absorption of compounds occurs through contact with the conjunctival and nasal mucosae, the latter occurring as the result of drainage through the nasolacrimal duct. Formulations of a gene delivery vehicle in inert ingredients such as buffers, chelating agents, antioxidants, and preservatives can be incorporated into ophthalmic dosage forms intended for multiple dose use. Formulations also may consist of aqueous suspensions, ointments, gels, inserts, bioadhesives, microparticles, and nanoparticles.

The nasal cavity also offers an alternative route of administration for a gene delivery vehicle. The human nasal cavities have a total surface area of approximately 150 cm^2 and are covered by a highly vascular mucosal layer. A respiratory epithelium comprised of columnar cells, goblet cells, and ciliary cuboidal cells line most of the nasal cavity (Chien et al., *Crit. Rev. in Therap. Drug Car. Sys. 4*:67-194, 1987). The subepithelia contains a dense vascular network and the venous blood from the nose passes directly into the systemic circulation, avoiding first-pass metabolism in the liver. Thus, delivery to the upper region of the nasal cavity may result in slower clearance and increased bioavailability. The absence of cilia in this area is an important factor in the increased effectiveness of nasal sprays as compared to drops. The addition of viscosity-building agents such as methycellulose can change the pattern of deposition and clearance of intranasal applications. Additionally, bioadhesives can be used as a means to prolong residence time in the nasal cavity. Various formulations comprising sprays, drops, and

powders, with or without the addition of absorptive enhancers have been investigated (*see* Wearley, *supra*).

Oral administration of a gene delivery vehicle includes sublingual, buccal, and gastrointestinal delivery. Sublingual and buccal (cheek) delivery allow for rapid systemic absorption of compounds which bypass hepatic first-pass metabolism and degradation in the stomach and intestines. Buccal delivery devices can be designed unidirectional for oral mucosal absorption only. Additionally these devices can prevent diffusion-limiting mucus buildup to allow for enhanced absorption. Delivery through the gastrointestinal track allows for precise targeting for drug release. Depending on the formulation, genes can be specifically delivered to areas in the stomach, duodenum, jejunum, ileum, cecum, colon, or rectum. Oral formulations include tablets, capsules, aqueous suspensions, and gels. These may contain bioadhesive polymers, hydrodynamically balanced systems, gastroinflatable delivery devices, intragastric retention shapes, enteric coatings, excipients, or intestinal sorption promoters (Ritschel, *Meth. Find. Exp. Clin. Pharmacol. 13*:313-336, 1991).

The human rectum has a surface area of between 200 to 400 cm^2 and is abundant in blood and lymphatic vessels. This offers an alternative route of administration of a gene delivery vehicle. Depending on the actual site of administration, it may be possible to bypass first-pass metabolism by the liver. Targeting of the systemic circulation can be achieved by delivering the vehicle to an area behind the internal rectal sphincter. This allows absorption directly into the inferior vena cava thereby bypassing the portal circulation and avoiding metabolism in the liver. The liver can be targeted by delivering the vehicle to the region of the ampulla recti which allows absorption into the portal system (Ritschel, *supra*).

Pulmonary administration of gene delivery vehicles can be accomplished through aerosolization. This allows systems to be delivered directly to the required site of action, minimizing systemic dilution and the dose administered. Systemic side-effects are also reduced, especially if the drug is locally metabolized. The three systems commonly used for aerosol production are: the nebulizer, the pressurized metered dose inhaler, and the dry powder inhaler. Aerosol therapy is very common in obstructive bronchial diseases but can be used for topical treatment of the respiratory tract as well as the treatment of systemic diseases (Kohler, *Lung Suppl.*:677-684, 1990). The surface area of the lung is approximately 75 m^2 and requires only one puff of an aerosol to cover this entire area within seconds. Absorption occurs quickly because the walls of the alveoli in the deep lung are extremely thin. Absorption and clearance depends on a number of factors including particle size and solubility (Wearley, *supra*).

Gene delivery vehicles can also be administered into the urinary bladder via catheterization (Morris et al., *J. of Urol. 152*:506-509, 1994). This intravesical therapy can be used for the treatment of bladder cancer. The rationale for intravesical therapy is that high concentrations of drug may contact tumor-bearing mucosa for prolonged periods. The likelihood of tumor implantation after resection maybe reduced by destroying viable cancer cells, as well as providing a cytotoxic effect on residual carcinoma. There is also minimal toxicity owing to limited systemic absorption. Additionally, the progression of disease requiring more intense local therapy, cystectomy, or systemic chemotherapy may be delayed or prevented (Herr et al., *J. of Urol 138*:1363-1368, 1987). Administration through catheterization is a relatively easy procedure accomplished by those skilled in the art.

The vaginal mucosa consists of stratified squamous epithelium. Gene delivery vehicles can be administered through the vaginal orifice onto the mucosa. Formulations include ointments, creams, and suppositories.

As noted above, gene delivery vehicles of the present invention may also be manipulated by a variety of methods known in the art, in order to render the expressed substance more immunogenic. Representative examples of such methods include: adding sequences that correspond to T helper epitopes; promoting cellular uptake by adding sequences encoding hydrophobic residues; or any combination of these (*see generally*, Hart, op. cit., Milich et al., *Proc. Natl. Acad. Sci. USA 85*:1610-1614, 1988; Willis, *Nature 340*:323-324, 1989; Griffiths et al., *J. Virol. 65*:450-456, 1991).

Pharmaceutical Compositions

Within preferred embodiments of the present invention, pharmaceutical compositions are provided comprising one of the above-described gene delivery vehicles, such as a recombinant retrovirus or recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus, herpes virus, SV40, HIV, measles, corona and Sindbis virus in combination with a pharmaceutically acceptable carrier or diluent. The composition may be prepared either as a liquid solution, or as a solid form (*e.g.*, lyophilized) which is suspended in a solution prior to administration. In addition, the composition may be prepared with suitable carriers or diluents for surface administration, injection, oral, or rectal administration. Generally, the recombinant virus is utilized at a concentration ranging from 0.25% to 25%, and preferably about 5% to 20% before formulation. Subsequently, after preparation of the composition, the gene delivery vehicle will constitute about 1 ug of material per dose, with about 10 times this amount material

(10 ug) as copurified contaminants. Preferably, the composition is prepared in 0.1-1.0 ml of aqueous solution formulated as described below.

Pharmaceutically acceptable carriers or diluents are nontoxic to recipients at the dosages and concentrations employed. Representative examples of carriers or diluents for injectable solutions include water, isotonic saline solutions which are preferably buffered at a physiological pH (such as phosphate-buffered saline or Tris-buffered saline), mannitol, dextrose, glycerol, and ethanol, as well as polypeptides or proteins such as human serum albumin. A preferred composition comprises a recombinant virus in a 10 mg/ml mannitol, 1 mg/ml HSA, 20 mM Tris, pH 7.2 and 150 mM NaCl. In this case, since the gene delivery vehicle represents approximately 1 ug of material, it may be less than 1% of high molecular weight material, and less than 1/100,000 of the total material (including water). This composition is stable at -70°C for at least six months. The composition may be injected intravenously (i.v.) or subcutaneously (s.c.), although it is generally preferable to inject it intramuscularly (i.m.). The individual doses normally used are 10^6 to 10^9 c.f.u. (colony forming units of neomycin resistance titered on HT1080 cells). These are generally administered at one- to four-week intervals for three or four doses initially. Subsequent booster shots may be given as one or two doses after 6-12 months, and thereafter annually.

Oral formulations may also be employed with carriers or diluents such as cellulose, lactose, mannitol, poly(DL-lactide-co-glycolate) spheres, and/or carbohydrates such as starch. The composition may take the form of, for example, a tablet, gel capsule, pill, solution, or suspension, and additionally may be formulated for sustained release. For rectal administration, preparation of a suppository may be accomplished with traditional carriers such as polyalkalene glucose, or a triglyceride.

Pharmaceutical compositions of the present invention may also include factors which stimulate cell division, and hence, uptake and incorporation of a recombinant retroviral vector. Representative examples include melanocyte stimulating hormone (MSH), for melanomas or epidermal growth factor (EGF) for breast or other epithelial carcinomas.

Particularly preferred methods and compositions for preserving recombinant viruses are described in a U.S. application entitled "Methods for Preserving Recombinant Viruses".

As noted above, the gene delivery vehicle may direct the expression of an immunomodulatory factor in addition to at least one substance. If the gene delivery vehicle, however, does not express an immunomodulatory factor which is a cytokine, this cytokine may be included in the above-described compositions, or may be administered separately (concurrently or subsequently) with the above-described compositions. Briefly, within such an embodiment, the immunomodulatory factor is preferably administered

according to standard protocols and dosages as prescribed in *The Physician's Desk Reference*. For example, alpha interferon may be administered at a dosage of 1-5 million units/day for 2-4 months, and IL-2 at a dosage of 10,000-100,000 units/kg of body weight, 1-3 times/day, for 2-12 weeks. Gamma interferon may be administered at dosages of 150,000-1,500,000 units 2-3 times/week for 2-12 weeks.

The following examples are offered by way of illustration and not by way of limitation.

EXAMPLES

EXAMPLE 1

Mice were injected three times at weekly intervals at multiple sites or at a single site with a representative gene delivery vehicle, N2 IIIB env (10^6 CFU/ml). Specifically, ten mice were injected at two intramuscular sites (right and left hind flank muscles) and ten mice were injected at one intramuscular site (right hind flank muscle). After seven days, a CTL assay (Warner et al., *AIDS Res. and Hum. Retroviruses* 7(8):645-655, 1991) was performed on each animal. Figure 1A depicts the results of the CTL assay for animals injected at multiple sites; Figure 1B representing CTL assay results for animals injected at a single site. Each bar within Figures 1A and 1B represents the percent (%) net target cell lysis associated with an individual mouse. As illustrated in Figure 1A, nine out of ten mice exhibited target cell lysis of greater than 60% (average of 76%) at an effector:target ratio of 100:1. By comparison, as illustrated in Figure 1B, only three out of ten mice achieved 60% target cell lysis at a effector:target ratio of 100:1 (average of 39%).

EXAMPLE 2

Within this experiment, five mice were injected two times at a weekly interval at multiple sites. Specifically, two intramuscular sites (left- and right-hand limbs) were injected with N2 IIIB env that was diluted 1:2 (0.5X concentration). A separate group of five mice were injected two times at a weekly interval at one site (right hind limb) with N2 IIIB env at a concentration of 1X. The two groups of mice received identical vector particle doses.

The results of a CTL assay (Warner et al., *supra*) are depicted in Figure 2. As shown in Figure 2A, mice receiving the gene delivery vehicle at multiple sites generated, on average, a higher target cell lysis via CTL (30.6%) than mice receiving the same quantity of vector at a single site (15%).

From the foregoing, it will be appreciated that, although specific embodiments of the invention have been described herein for purposes of illustration, various modifications may be made without deviating from the spirit and scope of the invention. Accordingly, the invention is not limited except as by the appended claims.

Claims

1. A method of stimulating an immune response within a warm-blooded animal, comprising:

administering to the animal at multiple sites at least one gene delivery vehicle, each of the gene delivery vehicles directing the expression of at least one substance in host cells modified with the vehicle, the substance(s) being capable of stimulating an immune response within the animal.

2. The method of claim 1 wherein the gene delivery vehicle is a recombinant retrovirus.

3. The method of claim 1 wherein the gene delivery vehicle is a Sindbis virus.

4. The method of claim 1 wherein the gene delivery vehicle is a recombinant virus selected from the group consisting of poliovirus, rhinovirus, pox virus, canary pox virus, vaccinia virus, influenza virus, adenovirus, parvovirus, adeno-associated virus, herpes virus, SV40, HIV, measles and corona virus.

5. The method of claim 1 wherein the substance or substances stimulate a cell-mediated immune response.

6. The method of claim 1 wherein the substance or substances stimulate both an HLA class I and class II-restricted immune response.

7. The method of claim 1 wherein at least one substance is an HIV antigen.

8. The method of claim 7 wherein the HIV antigen is selected from the group consisting of gp160, gp120 and gp41 antigens.

9. The method of claim 1 wherein at least one substance is a feline immunodeficiency virus antigen.

10. The method of claim 9 wherein the FIV antigen is selected from the group consisting of p15gag, p24gag, p10gag, p13pol, p62pol, p15pol and p36pol antigens.

11. The method of claim 9 wherein the FIV antigen is selected from the group consisting of gp68env, gp27env and rev antigens.

12. The method of claim 1 wherein at least one substance is an FeLV antigen.

13. The method of claim 12 wherein the FeLV antigen is selected from the group consisting of p15gag, p12gag, p27gag, p10gag, p14pol, p80pol, p46pol, gp70env and p15env antigens.

14. The method of claim 12 wherein the FeLV antigen is gp85env.

15. The method of claim 1 wherein at least one substance is a hepatitis antigen.

16. The method of claim 15 wherein the hepatitis antigen is selected from the group consisting of HBeAg, HBcAg, HBsAg, ORF 5, ORF 6 and HBV pol antigens.

17. The method of claim 1 wherein at least one substance is antigen X.

18. The method of claim 1 wherein at least one substance is an immunogenic, non-tumorigenic form of an altered cellular component.

19. The method of claim 18 wherein the altered cellular component is selected from the group consisting of ras*, p53*, Rb*, ubiquitin*, mucin*, DCC, APC, MCC and neu.

20. The method of claim 1 wherein the gene delivery vehicles are administered via intramuscular injection.

21. A method of suppressing an immune response within a warm-blooded animal, comprising:

administering to the animal at multiple sites at least one gene delivery vehicle, each of the gene delivery vehicles directing the expression of at least one substance in host cells modified with the vehicle, the substance(s) being capable of suppressing an immune response within the animal.

22. The method of claim 21 wherein at least one substance is an allergen.

23. The method of claim 22 wherein the allergen is selected from the group consisting of casein, glutin and bee venom.

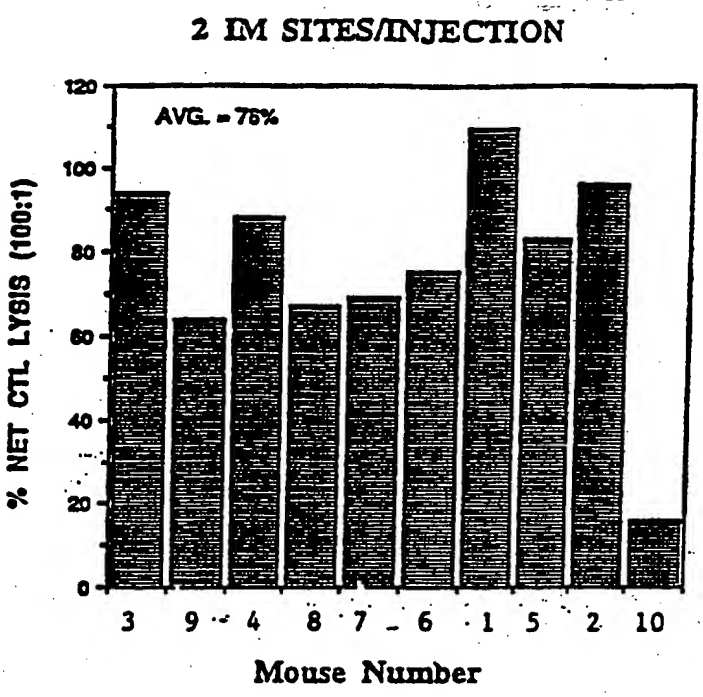
24. The method of claim 21 wherein at least one substance is an autoantigen.

25. The method of claim 21 wherein the gene delivery vehicle is a recombinant retrovirus.

26. The method of claim 21 wherein the gene delivery vehicle is a Sindbis virus.

Figure 1

1A



1B

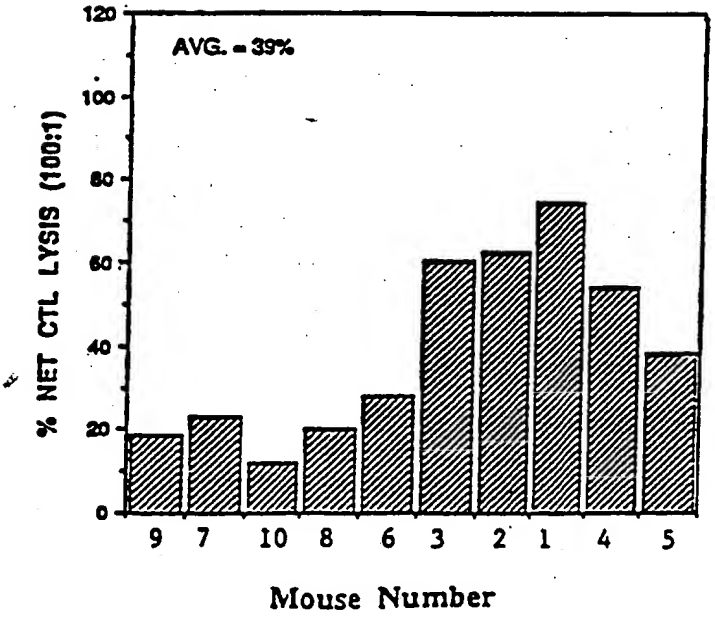
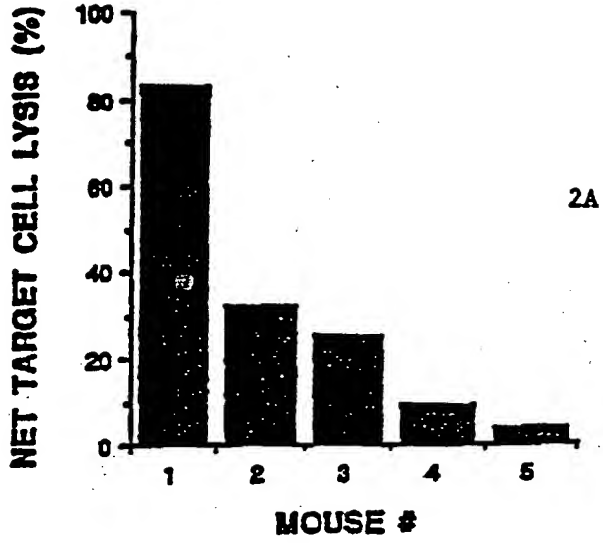
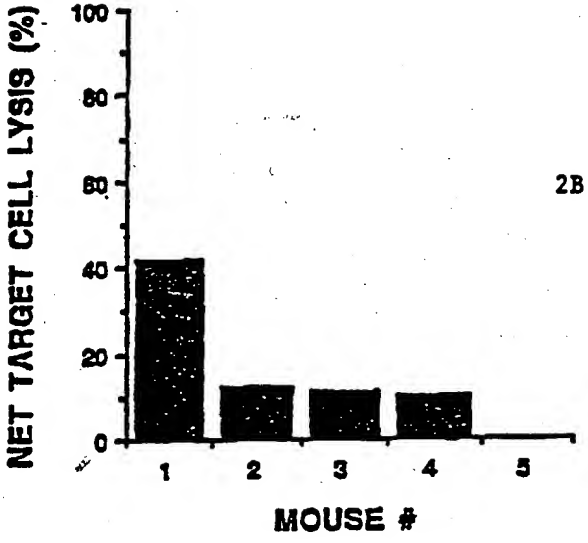


FIGURE 2



2A



2B

INTERNATIONAL SEARCH REPORT

Internation pplication No
PCT/US 95/16471

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 A61K48/00 //C12N15/86

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 A61K C12N

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	JOURNAL OF VIROLOGY, vol. 68, August 1994, pages 5036-5044, XP002003656 IRWIN, M.J. ET AL.:	1-8
Y	see page 5037, right-hand column, line 36 - line 66	9-20

Y	KEYSTONE SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, JOURNAL OF CELLULAR BIOCHEMISTRY SUPPL. 18A. 1994, JANUARY 4-23, 1994, page 220 XP002003657 JOLLY, D.J. ET AL.: cited in the application see abstract	9-20

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[X] Further documents are listed in the continuation of box C.

[X] Patent family members are listed in annex.

* Special categories of cited documents :

"A" document defining the general state of the art which is not considered to be of particular relevance

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"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art.

"&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
22 May 1996	07.06.96

Name and mailing address of the ISA	Authorized officer
European Patent Office, P.B. 5818 Patentlaan 2 NL - 2280 HV Rijswijk Tel. (+31-70) 340-2040, Tx. 31 651 epo nl, Fax (+31-70) 340-3016	Olsen, L

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C.(Continuation) DOCUMENTS CONSIDERED TO BE RELEVANT

Category *	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	KEYSTONE SYMPOSIA ON MOLECULAR & CELLULAR BIOLOGY, JOURNAL OF CELLULAR BIOCHEMISTRY SUPPL. 17E, 1993, MARCH 29- APRIL 25, 1993, page 79 XP002003658 IRWIN, M.J. ET AL.: ---	1-8
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A	WO,A,91 02805 (VIAGENE, INC.) 7 March 1991 see page 38, line 16 - page 39, line 4 ---	1-26
A	WO,A,93 00051 (THE REGENTS OF THE UNIVERSITY OF MICHIGAN) 7 January 1993 cited in the application see the whole document ---	1-26
A	WO,A,90 11092 (VICAL, INC.) 4 October 1990 cited in the application see the whole document -----	1-26

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Internati Application No
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		AU-B-	648261	21-04-94
		AU-B-	6185390	03-04-91
		CA-A-	2066053	19-02-91
		EP-A-	0487587	03-06-92
		JP-T-	4507196	17-12-92
WO-A-9300051	07-01-93	US-A-	5328470	12-07-94
		CA-A-	2112375	07-01-93
		CA-A-	2112376	07-01-93
		EP-A-	0591385	13-04-94
		EP-A-	0591408	13-04-94
		JP-T-	6509328	20-10-94
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		CA-A-	2049287	22-09-90
		EP-A-	0465529	15-01-92
		JP-T-	4504125	23-07-92